The Envelope Glycoproteins of Gammaretroviruses and Type-D Betaretroviruses are Tetherin Antagonists:

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The Envelope Glycoproteins of Gammaretroviruses and Type-D Betaretroviruses are Tetherin Antagonists

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Tetherin/BST2 is an interferon-inducible antiviral factor that restricts the egress of numerous enveloped viruses including HIV-1. Consequently, many viruses have evolved mechanisms to actively or passively evade restriction by tetherin. Most studies conducted to date focused on the tetherin-evasion mechanism of complex retroviruses like HIV and SIV, which encode accessory proteins like Vpu and Nef respectively to counteract tetherin-mediated restriction. However, there is a wide gap in knowledge in understanding how simple retroviruses (that includes alpharetroviruses, some betaretroviruses and gammaretroviruses) that lack obvious accessory proteins like HIV-1 Vpu and SIV-Nef, evade restriction by tetherin.

In this dissertation, I have established that Simian retrovirus type-3, a prototypical type-D betaretrovirus, isolated from Asian macaques, is restricted by human tetherin but not by rhesus macaque tetherin. This differential sensitivity indicated that SRV-3 has a mechanism to evade tetherin-mediated restriction. I have identified the SRV-3 envelope (Env) glycoprotein as the viral determinant of tetherin antagonism, and have also found that SRV-3 envelope expression *in-trans* was sufficient to rescue a heterologous virus from tetherin. SRV-3 Env resulted in cell-surface down-modulation of rhesus tetherin, and this mechanism of tetherin-antagonism is independent of the SRV-3 Env trafficking pathway. The target specificity of SRV-3 Env overlapped a stretch of five residues

($G_{14}DIWK_{18}$) in the rhesus tetherin cytoplasmic tail that are absent from human tetherin. Additionally, I was able to show that SRV-3 Env physically interacts with rhesus tetherin by targeting the $G_{14}DIWK_{18}$ motif.

SRV-3 belongs to a large supergroup of retroviruses, called the RDR Interference Supergroup. Due to this reason, I screened additional RDR envelope glycoproteins for their ability to antagonize a panel of tetherin homologs. All the RDR envelopes tested were sensitive to human tetherin but exhibited anti-tetherin activity when tested against a panel of tetherin homologs from squirrel monkey, baboon, dog and cat.

I also found that several non-RDR gammaretroviral envelope glycoproteins also have anti-tetherin function. Thus, tetherin-antagonism is not just restricted to the envelope glycoproteins of retroviruses in the RDR interference supergroups but extends to other non-RDR gammaretroviruses as well. To my knowledge, this is the first characterization of gamma-type envelopes as tetherin antagonists. Thus, in the absence of a dedicated tetherin antagonist, many simple retroviruses in the beta- and gammaretrovirus genera may evade tetherin-mediated restriction through neo-functionalization of their envelope glycoproteins. We speculate that the evolutionary success of the gamma-type envelope may be due, at least in part, to this anti-tetherin function.

This dissertation is dedicated to my Ma and Baba (my parents) and my husband without whose constant support and encouragement this would not have been possible.

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LIST OF ABBREVIATIONS

A	alanine
Å	angstrom
AIDS	acquired immunodeficiency syndrome
ALV	avian leucosis virus
APOBEC3	APOlipoprotein B Editing Catalytic subunit-like 3
Art-tetherin	artificial tetherin
ASCT2	alanine, serine, cysteine- preferring transporter 2
BaEV	baboon endogenous retrovirus
BFV	bovine foamy virus
BLV	bovine leukemia virus
BST2	Bone Marrow Stromal Antigen 2
^{0}C	degree Celsius
CA	capsid protein
CaEV	Caprine arthritis encephalitis virus
CAML	calcium-modulating ligand
CCR5	C-C chemokine receptor type 5
CD4	cluster of differentiation 4
CD28	cluster of differentiation 28
CD34	cluster of differentiation 34
CD317	cluster of differentiation 317
cDNA	complementary deoxyribonucleic acid
CoeEFV	coelacanth endogenous foamy-like virus
CTEs	constitutive transport elements
CXCR4	CXC receptor 4
CT	cytoplasmic tail
D	aspartic acid
dNTP	deoxynucleotide triphosphate
DMEM	Dulbecco's Modified Eagle's medium
DMPK	dystrophia myotonica protein kinase
DNA	deoxyribonucleic acid
E	glutamic acid

EC	ectodomain
EFV	equine foamy virus
eGFP	enhanced green fluorescent protein
ER	endoplasmic reticulum
EIAV	equine infectious anemia virus
env	envelope gene
Env	envelope glycoprotein
F	fusion protein
FeLV	feline leukemia virus
FFV	feline foamy virus
FBS	fetal bovine serum
G	glycine
GaLV	gibbon ape leukemia virus
gag	group specific antigen gene
GFP	green fluorescent protein
GPI	glycosyl-phosphatidylinositol
FIV	feline immunodeficiency virus
HCoV	human coronavirus
HEK-293T/17	Human Embryonic Kidney 293T/17 cells
HERV-K	human endogenous retrovirus type K
HERV-W	human endogenous retrovirus type W
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HN	hemagglutinin-neuraminidase protein
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HTLV	human-T lymphotropic virus
I	isoleucine
IFN	interferons
IFITMs	interferon-induced transmembrane proteins
IL-27	interleukin-27
ISD	immunosuppressive domain
JSRV	Jaagsiekte sheep retrovirus
K	lysine
kDa	kilodalton
KSHV	Kaposi's sarcoma herpes virus
L	leucine
LTR	long terminal repeat
MA	matrix protein
MHC	major histocompatibility complex

MLV	murine leukemia virus
MMTV	mouse mammary tumor virus
MPMV	Mason-Pfizer monkey virus
mRNA	messenger RNA
MSD	membrane spanning domain
Mx2	human myxovirus resistance 2
N	asparagine
NA	neuraminidase protein
NC	nucleocapsid
nef	negative regulatory factor gene
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NS1	non-structural protein 1 of influenza virus
NWM	New world monkey
ORF	open reading frame
OWM	Old world monkey
PACS	phosphofurin acid cluster sorting proteins
PBMCs	peripheral blood mononuclear cells
PHV	perch hyperplasia retrovirus
PIC	pre-integration complex
pbs	primer binding site
ppt	polypurine tract
pol	polymerase gene
Pol	polymerase protein
poly (A)	polyadenylation
pro	protease gene
Pro	protease protein
PRRs	pattern recognition receptors
pSIVgml	gray mouse lemur prosimian immunodeficiency virus
RER	rough endoplasmic reticulum
REV	reticuloendotheliosis virus
rev	regulator of virion gene
RFs	restriction factors
RNA	ribonucleic acid
rpm	revolutions per minute
RSV	Rous sarcoma virus
RT	reverse transcriptase
S	serine
sag	superantigen gene
S	serine
SAMHD1	sterile alpha motif and histidine-aspartic domains containing protein 1

SFV	simian foamy virus
SFVcpz (hu)	simian foamy virus isolate of chimpanzees
SIVagm	simian immunodeficiency virus of African green monkeys
SIVcpz	simian immunodeficiency virus of chimpanzees
SIVgor	simian immunodeficiency virus of gorilla
SIVmac	simian immunodeficiency virus of rhesus macaques
SIVrcm	simian immunodeficiency virus of red-capped monkey
SIVsmm	simian immunodeficiency virus of sooty mangabeys
SIVtan	simian immunodeficiency virus of tantalus monkeys
SMRV	squirrel monkey retrovirus
SnRV	snakehead retrovirus
SRV-1	simian retrovirus type 1
SRV-2	simian retrovirus type 2
SRV-3	simian retrovirus type 3
SRV-4	simian retrovirus type 4
SRV-5	simian retrovirus type 5
SRV-6	simian retrovirus type 6
SRV-7	simian retrovirus type 7
SRV-8	simian retrovirus type 8
SSSV	salmon swimbladder sarcoma virus
STLV	simian T-lymphotropic virus
SU	surface
T	threonine
tat	trans-activator of transcription gene
TfR	transferrin receptor
TGN	trans-Golgi network
TLR	toll-like receptor
TM	transmembrane
Trim-5α	Tripartite motif 5-alpha
UTR	untranslated region
uPAR	urokinase plasminogen activator receptor
V	valine
vif	viral infectivity factor gene
vpu	viral protein u gene
vpr	viral protein r gene
vpx	viral protein x gene
VS	virological synapse
VSV	vesicular stomatitis virus
VSV-G	vesicular stomatitis virus glycoprotein
W	tryptophan

WDSV	Walleye dermal sarcoma virus
WEHV	Walleye epidermal hyperplasia virus
Y	tyrosine

CHAPTER 1: INTRODUCTION

1.1 Retroviruses

Retroviruses are highly pathogenic enveloped viruses that have single-stranded positive-sense RNA genomes. These viruses have been isolated from diverse vertebrate hosts, including fishes, birds, reptiles and mammals.[1] The most distinguishing feature of a retrovirus is the presence of the enzyme viral reverse transcriptase (RT), that converts the viral RNA genome into double-stranded DNA.[2] This viral DNA gets irreversibly integrated into the host cell genome by the viral integrase (IN) to form a DNA provirus.[2]

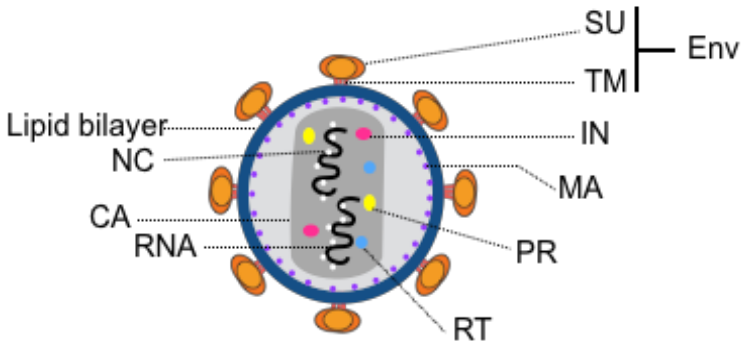
A mature retrovirus ranges between 80nm-100nm in diameter and is enclosed by the host cell-derived lipid bilayer.[2] The retrovirus consists of a dimeric positive-sense RNA genome that has a 5' cap and a long poly (A) tail at its 3' end.[3] The viral RNA genome is flanked by a short-repeated region (R) on either side. A unique 5' sequence (U5) important for proviral integration is situated downstream of the 5' R region. The primer binding site (pbs) is located right after the U5 region.[4] Another unique 3' sequence (U3) is situated downstream of the retroviral genes. The U3 sequence is preceded by a short polypurine tract (ppt). The ppt serves as the initiation site for the synthesis of the DNA sense strand.[4] A quintessential retroviral genome consists of four genes namely *gag*, *pro*, *pol*, and *env*.[4] The *gag* encodes the viral structural proteins: matrix (MA), capsid (CA) and nucleocapsid (NC). The *pro* and *pol* genes encode the viral enzymes: protease (PR), integrase (IN) and reverse transcriptase (RT). The *env* gene encodes an envelope glycoprotein (Env) that is characterized by a surface subunit (SU) and a transmembrane subunit (TM). The SU and the TM subunits oligomerizes to form heterotrimers that appear as spikes embedded in the lipid bilayer envelope (Figure 1.1A).

The functions of the different viral proteins in the retroviral life cycle are summarized in table 1.1.

Retroviruses are often referred to as simple or complex on the basis of the presence or absence of accessory genes. Alpharetroviruses, gammaretroviruses and some betaretroviruses encode the four essential retroviral genes (*gag*, *pro*, *pol* and *env*), and lack accessory genes; hence, are termed as simple retroviruses (Figure 1.1B). Lentiviruses, deltaretroviruses, epsilonretroviruses and spumaviruses are called complex retroviruses as they have additional accessory genes in their genomes (Figure 1.1B).

Depending on the mode of transmission, retroviruses are also classified as exogenous or endogenous retroviruses. Retroviruses that are horizontally transmitted from one individual to another are known as exogenous retroviruses. Occasionally, a retrovirus infects a germ line cell and randomly integrates its genome into the host genome to form a provirus that may persist and get vertically transmitted from parent to offspring. Such a retroviral insertion is called endogenous retrovirus.[5] Majority of the retroviruses are well-known for their pathogenicity, but some of them also serve as powerful tools for gene delivery.[6]

(A)



(B)

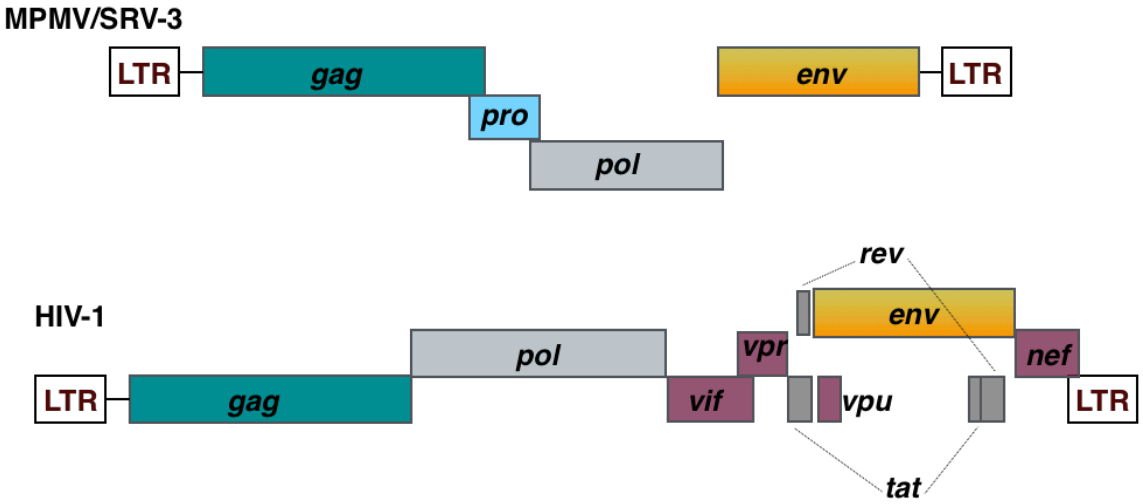


Figure 1.1. Retrovirus virion and genomes. (A) Schematic showing the structure of a mature retrovirus. The matured virion is surrounded by a lipid bilayer (dark blue) derived from the host cell. The envelope heterotrimers are embedded in the lipid bilayer (orange). The matrix protein (MA; shown in purple) is situated underneath the lipid bilayer. The dimeric viral RNA genome and the viral enzymes reverse transcriptase (RT; light blue), protease (PR; yellow) and integrase (IN; pink) are enclosed within the capsid core (CA; grey). (B) Schematic showing the genomic organization of retrovirus. Cartoons showing the DNA proviruses from the simple retrovirus (MPMV/SRV-3) and complex retrovirus (HIV-1). MPMV has a simple genomic organization consisting of the *gag* (green), *pro* (blue), *pol* (light blue) and *env* (orange) genes. In contrast, the HIV-1 has a more complex genomic organization. Apart from the *gag* (green), *pol* (light blue; pro and pol are expressed from the same reading frame) and *env* (orange) genes, HIV-1 has two regulatory genes, *tat* and *rev* (gray) and accessory genes like *vif*, *vpu* and *nef* (dark pink). The long terminal repeats (LTRs) are shown on either side of the genomes. The LTRs have regulatory functions.

Table 1.1. The functional significance of the retroviral proteins.

Viral Proteins	Function
Matrix (MA)	Structural protein encoded by *gag*; helps in the assembly of retroviral particles at the plasma membrane.[2]
Capsid (CA)	Structural protein encoded by *gag*; forms a protective shell surrounding the inner viral core.[2]
Nucleocapsid (NC)	Structural protein encoded by *gag*; binds to the viral RNA and promotes viral genome packaging.[2]
Protease (PR)	Viral enzyme; promotes viral maturation by cleaving the Gag and Gag-Pol polyproteins into their respective viral proteins.[2]
Reverse Transcriptase (RT)	Viral enzyme; reverse transcribes the viral RNA genome into a ds-DNA.[2]
Integrase (IN)	Viral enzyme; favors integration of the viral DNA into the host genome.[2]
Surface subunit (SU)	Envelope glycoprotein subunit; recognizes and binds to the receptor on the host cell surface.[2]
Transmembrane subunit (TM)	Envelope glycoprotein subunit; mediates fusion with the host cell membrane and facilitates viral entry.[2]

*The number in the suffix indicates reference.

1.2 Life cycle of retrovirus

The retroviruses perpetuate through an extraordinary life cycle that involves reverse transcription and provirus formation. The entry of the retrovirus within the host cell is mediated by the recognition and the binding of the host cell receptor by the surface subunit of the viral envelope glycoprotein.[2] Different genera of retroviruses use different receptors for viral entry. For instance, HIV-1 uses an immunoglobin-like molecule called CD4 as a receptor and a co-receptor called CXCR4 or CCR5.[7] The gammaretroviruses use solute transporters with multiple transmembrane domains as receptors.[8] Upon binding to the host cell receptor, both the SU and TM undergo conformational changes which facilitate fusion with the host cell membrane. The fusion event leads to the release and uncoating of the viral capsid core in order to deliver the viral RNA genome and the viral proteins into the host cell cytoplasm. The viral RNA is reverse transcribed into double-stranded (ds) DNA in the cytoplasm. The viral DNA together with the cellular and the viral proteins forms the pre-integration complex (PIC). The PIC is imported into the nucleus, and the viral DNA is integrated randomly within the host genome leading to the formation of a DNA provirus.

The viral DNA is transcribed into unspliced, singly-spliced and multiple spliced mRNA transcripts that are exported out of the nucleus. The unspliced mRNAs form the viral genomic RNA and also encode the viral Gag and Gag-Pol precursor polyproteins in the cell cytoplasm. The unspliced mRNAs of type-D retroviruses contain cis-acting elements called the constitutive transport elements (CTEs).[9] The CTEs facilitate the nuclear export of unspliced mRNA transcripts. In complex retroviruses like HIV-1, nuclear export of unspliced mRNA is carried out by the regulator of expression of virion

(REV) protein.[4] The singly-spliced mRNA encodes the envelope glycoprotein (Env) in simple retroviruses whereas alternatively spliced mRNAs encode the Env and accessory proteins in complex retroviruses.[4] Unlike the Gag and Gag-Pol polyproteins, the envelope proteins are synthesized in the rough endoplasmic reticulum (RER) and are transported to the plasma membrane via the trans-Golgi network.

Most retroviruses assemble at the plasma membrane with the exceptions of beta- and spumaretroviruses that assemble in the cell cytoplasm.[4] Following assembly, the retroviral particle buds out from the host cell membrane and is released. During this step, the immature retroviral particle acquires the lipid bilayer envelope from the host cell. Eventually, the viral protease cleaves the Gag and the Gag-Pol precursor polyproteins into their respective proteins to produce a mature infectious virion. The different stages in the retroviral life cycle are illustrated in Figure 1.2.

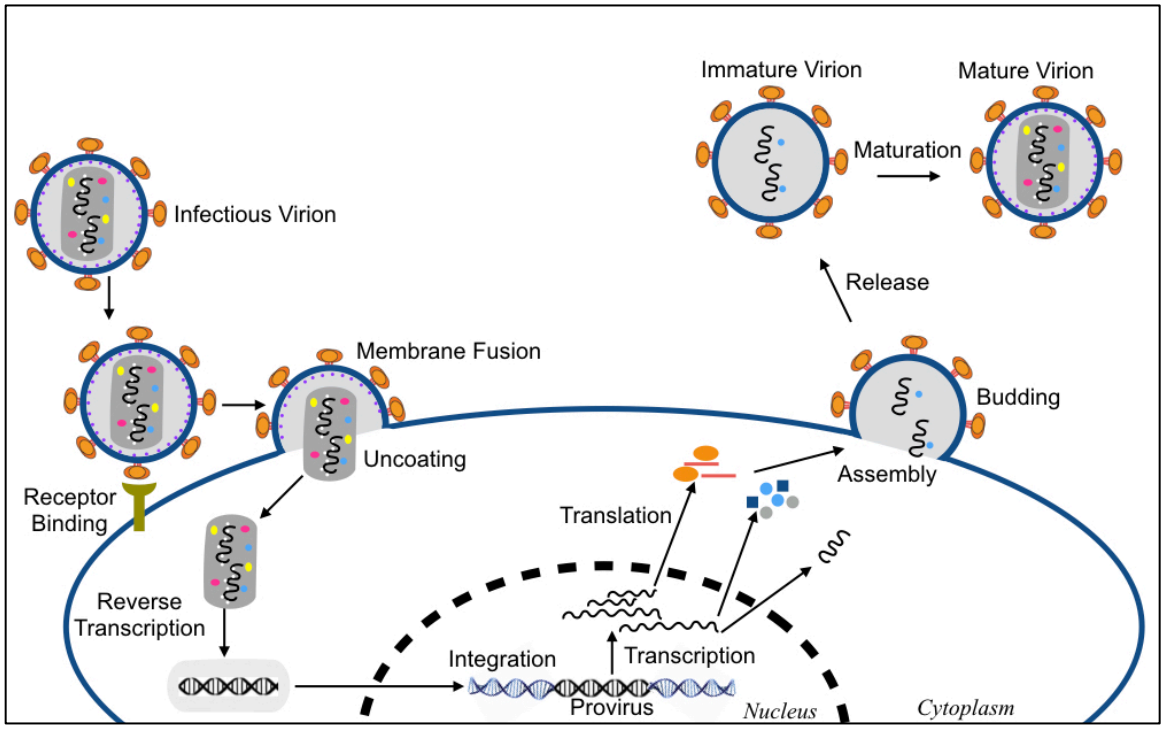


Figure 1.2. Schematic illustrating the life cycle of a retrovirus. The retroviral life cycle consists of the following steps: receptor binding, membrane fusion, uncoating, reverse transcription of viral RNA to ds-DNA, nuclear import and integration of pre-integration complex to form a provirus, transcription of the viral DNA, nuclear export and translation, viral assembly, virion budding, release, maturation and re-infection.

1.3 Taxonomic classification of retrovirus

The family *Retroviridae* is classified into two subfamilies: the *Orthoretrovirinae* and the *Spumaretrovirinae*.[4] The *Orthoretrovirinae* consists of six genera: alpharetrovirus, betaretrovirus, gammaretrovirus, deltaretrovirus, epsilonretrovirus and lentivirus.[4] The *Spumaretrovirinae* consists of a single genus, spumavirus.[4] A phylogenetic relationship among the retroviruses of the different genera of the *Orthoretrovirinae* and the *Spumaretrovirinae* subfamilies are shown in Figure 1.3. The distinguishing features of the retroviruses belonging to the different genera are briefly discussed below and are summarized in Table 1.2.

1.3.1 Subfamily: *Orthoretrovirinae*

1.3.1.1 Genus: Alpharetrovirus

As discussed in section 1.1, alpharetroviruses are considered as simple retroviruses since their genomes contains ORFs for only four genes: *gag*, *pro*, *pol* and *env*. Presence of any accessory genes has not yet been reported. The alpharetroviruses exhibit a 'C-type' morphology meaning that they all have round and centrally located nucleocapsid cores.[2] The viral assembly takes place at the plasma membrane.[10] This genus consists of both oncogenic and non-oncogenic exogenous and endogenous retroviruses of avian species. Two well-known examples of alpharetroviruses are the Rous sarcoma virus and Avian Leukosis virus. Rous sarcoma virus was first identified as an oncovirus by Peyton Rous in 1911. This discovery was very significant as it opened the gateway to the discovery of numerous other retroviruses.

1.3.1.2 Genus: Betaretrovirus

Like alpharetroviruses, betaretroviruses include both simple exogenous and endogenous retroviruses exhibiting an eccentric and spherical ('B-type' morphology) or a cylindrical nucleocapsid core ('D-type' morphology).[10] These retroviruses assemble their Gag proteins into immature capsid-like particles in the cytoplasm that are transported to the plasma membrane for budding and release.[10] The betaretroviral genome consists of *gag*, *pro*, *pol* and *env* genes, all of which are in different reading frames (Figure 1.1B).[10]

The type-B betaretroviruses are characterized by a beta-type envelope TM subunit whereas the type-D retroviruses are regarded as recombinant betaretroviruses because their envelope glycoproteins consist of gamma-type TM subunits and resemble the envelope glycoprotein of a typical gammaretrovirus.[11] The different types of retroviral envelopes are discussed in details in section 1.4. Evidence suggests that the type-D retroviruses originated as a result of a recombination between an ancient virus containing beta-like viral *gag* and *pol* genes, and an ancient virus containing a gamma-like viral *env* gene.[11] As such, the type-D retroviruses share a high degree of sequence homology in their Gag and Pol proteins with the betaretroviruses but their *env* gene resembles that of gammaretroviruses.[12] The type-D retroviruses consist of eight serotypes of simian retroviruses (SRV-1 through SRV-8), all of which have been isolated from Asian macaques excepting SRV-6/Po1-Lu, which has been isolated from langurs.[13,14,15] This group also includes a new world squirrel monkey retrovirus (SMRV).[16] The prototypical member of the type-D retrovirus, the Mason-Pfizer monkey virus (also called SRV-3) was isolated from the mammary carcinoma of female rhesus macaques [17]. Similar to HIV and SIVs, SRVs also cause immunodeficiency syndromes in macaque species.[13,14]

The well-known examples of type-B exogenous retroviruses are Mouse mammary tumor virus (MMTV), causing breast cancer in mice and Jaagsiekte sheep retrovirus (JSRV), causing lung cancer in sheep. An example of a type-B endogenous retrovirus is HERV-K. Both MMTV and HERV-K are exceptions to this genus as they encode accessory proteins. The alternative splicing of the *env* gene in MMTV produces two accessory proteins- the superantigen (Sag) protein and the Rem protein.[18] The Rem protein helps in nuclear export of unspliced mRNA transcripts while the Sag protein helps in viral pathogenesis.[18] The HERV-K encodes an HIV-1 Rev-like nuclear export protein called Rec and a tumorigenesis protein called Np9.[19,20]

1.3.1.3 Genus: Gammaretrovirus

The gammaretrovirus genus comprises of the largest number of simple exogenous and endogenous retroviruses that are found in diverse vertebrates including mammals, birds, reptiles and amphibians. The gammaretroviruses have a C-type morphology, and their genomes consist of the *gag*, *pro*, *pol* and *env* genes only.[4] Presence of any accessory genes has not yet been reported in any members of this genus. The gammaretroviruses are usually associated with immunosuppression, neurological disorders and malignancies.[4] Some of the most prominent exogenous members of this genus are the murine leukemia virus (MLV), feline leukemia virus (FeLV), reticuloendotheliosis virus (REV), and gibbon ape leukemia virus (GaLV). Examples of two extensively studied endogenous gammaretroviruses are the baboon endogenous retrovirus (BaEV) and RD114.

1.3.1.4 Genus: Deltaretrovirus

The deltaretrovirus genus consists of only complex exogenous mammalian retroviruses characterized by a C-type morphology. Two notable members of this genus are the human T-lymphotropic virus (HTLV) and the bovine leukemia virus (BLV). Apart from the 4 essential retroviral genes, the deltaretroviruses also have two non-structural accessory genes called *tax* and *rex*.[10] The *rex* gene encodes for an analog of HIV-1 Rev protein called Rex.[21] Rex enhances the nuclear export of unspliced or minimally spliced mRNA transcripts.[21] The *tax* gene encodes for a transcriptional activator called Tax.[22] HTLV infection in humans are associated with leukemia/lymphomas and neurological disorders.

1.3.1.5 Genus: Epsilonretrovirus

The epsilonretrovirus has similar morphology like the deltaretrovirus and comprises of only complex exogenous piscine retroviruses. The prototypical member of this genus is the Walleye dermal sarcoma virus (WDSV). Other examples include snakehead retrovirus (SnRV), perch hyperplasia retrovirus (PHV) and salmon swimbladder sarcoma virus (SSSV). WDSV has three accessory genes- *orf a*, *orf b* and *orf c*.[23] The *orf a* gene encodes for a cell-cycle regulatory protein called the retroviral cyclin protein (rv-cyclin).[10,23,24] The *orf b* and *orf c* genes encode for Orf B and Orf C proteins having unknown functions.

1.3.1.6 Genus: Lentivirus

This genus consists of complex mammalian retroviruses either having a characteristic conical or cylindrical core.[10] Some famous examples of retroviruses of this

genus are the human immunodeficiency virus-1 and -2 (HIV-1 and -2), simian immunodeficiency viruses (SIVs), bovine immunodeficiency virus (BIV), equine infectious anemia virus (EIAV) and visna virus. Out of the above list, HIV-1 is well-known for causing acquired immunodeficiency syndrome (AIDS) and resulting in very high death rates worldwide. The 3' half of the HIV-1 genome contains several accessory genes- *vif*, *vpr*, *vpu*, *rev*, *tat* and *nef* (Figure 1.1B).[10] These accessory genes encode their respective proteins that perform a variety of functions during the viral replication cycle. All primate lentiviruses do not have identical sets of accessory genes. For example, HIV-2 and not HIV-1 has the *vpx* gene, and non-human primate lentiviruses like SIVs in rhesus macaques and sooty mangabeys lack the *vpu* gene.

1.3.2 Subfamily: *Spumaretrovirinae*

1.3.2.1 Genus: Spumavirus

The spumaretroviruses are also called foamy viruses because of their unique morphology. These complex retroviruses consist of a centrally positioned uncondensed core with the envelope glycoproteins forming spikes on the surface giving them a foamy appearance.[25] The virion assembly takes place within the cell cytoplasm, and the budding mostly occurs from the ER rather than the plasma membrane.[10] The first foamy virus was isolated from monkey kidney cells in 1955 and thereafter, several other foamy viruses were isolated from diverse mammalian species. The prototypical member, the human foamy virus was isolated from the nasopharyngeal carcinoma of a human patient in 1971. However, this virus shared nearly 85-95% sequence similarity with the simian foamy virus isolate of chimpanzees (SFVcpz), and so it was designated as SFVcpz(hu).[26] Apart

from the *gag*, *pro*, *pol* and *env* genes, the foamy virus genome also encodes two accessory genes called *tas* or *bel-1* and *bet*. The Tas protein acts as a transcriptional transactivator and initiates transcription from the LTR.[26] The exact function of *bet* gene is still unknown. However, one study suggested that the Bet protein encoded by the *bet* gene helps to counteract restriction by the host antiviral factor APOBEC3G.[27] Some prominent examples of spumaretroviruses are the simian foamy virus (SFV), equine foamy virus (EFV), feline foamy virus (FFV) and bovine foamy virus (BFV).

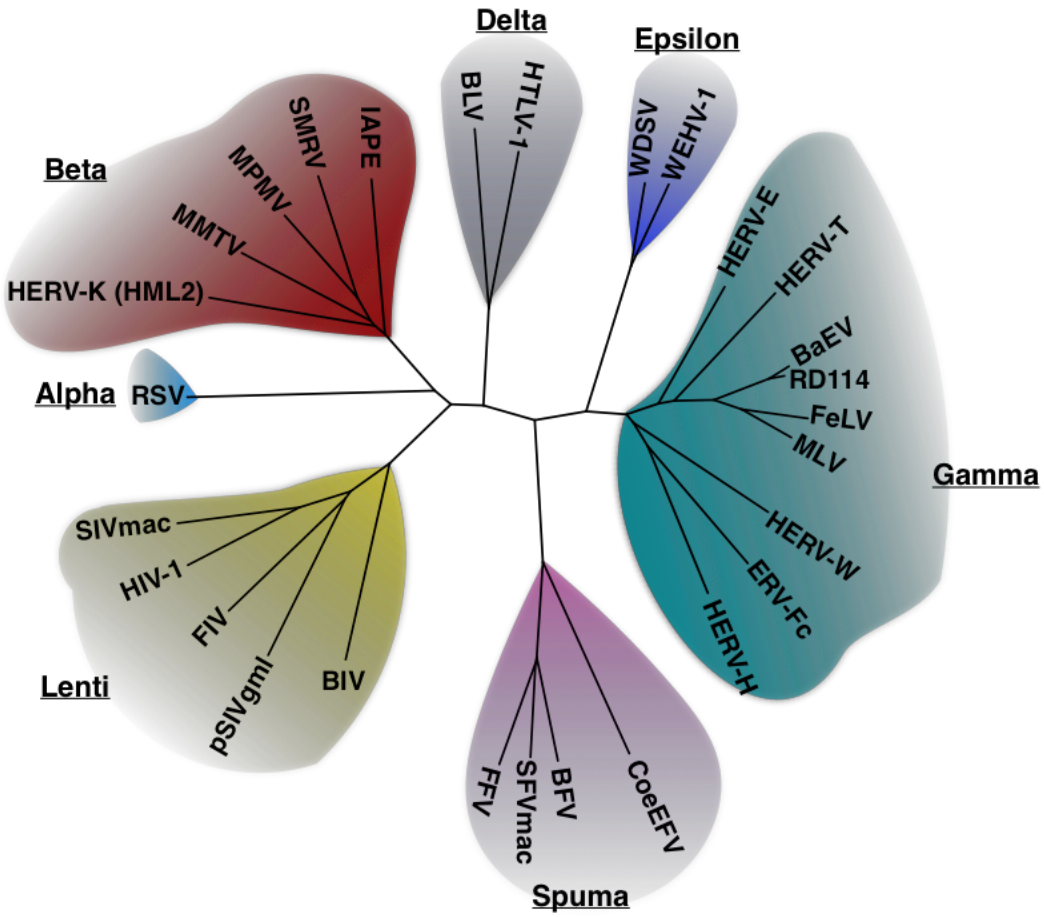


Figure 1.3. Phylogenetic relationship among the different retroviruses belonging to the *Orthoretrovirinae* and the *Spumaretrovirinae* subfamilies. The *Orthoretrovirinae* subfamily consists of the alpha-, beta-, gamma-, delta-, epsilon- and lentivirus. The *Spumaretrovirinae* consists of a single genus called the spumavirus. The phylogenetic tree is based on the polymerase sequences of some of the representative retroviruses belonging to the different genera.

Table 1.2. Showing the different genera of the *Orthoretrovirinae* and the *spumaretrovirinae* subfamilies, the salient features of the genera and a few examples of retroviruses belonging to the different retroviral genera.

Subfamily	Genus	Morphology	tRNA primer	Genome-type	Accessory genes	Examples
Orthoretrovirinae	Alpha	C-type	$tRNA^{Trp}$	Simple	none	RSV ALV
	Beta	B-type	$tRNA^{Lys-1,2}$ or $tRNA^{Lys-3}$	Simple	exceptions include *sag* and *rem* in MMTV *rec* in HERV-K	MMTV HERV-K JSRV
		D-type	$tRNA^{Lys-1,2}$ or $tRNA^{Lys-3}$	Simple	none	SRV-1 through SRV-8 SMRV
	Gamma	C-type	$tRNA^{Pro}$ or $tRNA^{Glu}$	Simple	none	MLV GaLV FeLV REV RD114 BaEV
	Delta	C-type	$tRNA^{Pro}$	Complex	rex and *tax*	STLV BLV HTLV
	Epsilon	C-type	$tRNA^{His}$ or $tRNA^{Arg}$	Complex	orf a, orf b and ofc c	WDSV WEHV PHV SnRV SSSV
	Lentivirus	Cylindrical or conical nucleocapsid core	$tRNA^{Lys-3}$	Complex	*vif, vpr, vpx, vpu, tat, rev and nef*	HIV-1 & HIV-2 SIVs FIV EIAV CAEV Visna virus
Spumaretrovirinae	Spumavirus	Uncondensed core	$tRNA^{Lys-1,2}$	Complex	*bet* and *tas/bel-1*	HFV SFV FFV BFV EFV

1.4 Retroviral envelope glycoprotein

The retroviral envelope (Env) glycoproteins are type-I membrane proteins and are synthesized as single precursor proteins on the RER from a spliced viral mRNA transcript. The first few amino acid residues on the N-terminus of the Env precursor protein constitutes a hydrophobic signal peptide, which cotranslationally inserts the Env protein into the lumen of the RER.[28] Once in the ER, the leader peptide is removed by the cellular protease, and the retroviral Env protein is heavily glycosylated and oligomerized into trimers. Glycosylation is necessary for proper protein folding and oligomerization ensures stable protein expression.[28] The Env trimers are exported to the Golgi and a furin-like cellular protease recognizes a polybasic residue (such as K/R-X-K/R-R) on the Env precursor and cleaves it into a C-terminal surface subunit (SU) and an N-terminal transmembrane subunit (TM).[11] Therefore, the resulting mature retroviral envelope glycoprotein is a heterotrimer (SU_3TM_3). The TM subunit consists of an ectodomain (protrudes outside the virion), a membrane-spanning domain (MSD) and a cytoplasmic tail (CT). The SU harbors the receptor binding domain and therefore determines host cell tropism, and the TM mediates fusion of the virion and the host cell membranes to facilitate viral entry.[11] In the case of simple retroviruses like MMTV and MLV, the cleavage of the viral envelope has been shown to be essential for the envelope fusogenicity during viral infection.[29,30] In MLV, the Env cleavage also facilitates anterograde trafficking and incorporation into viral particles.[31] The intracellular trafficking mechanisms of the retroviral envelopes are still unclear. However, one study suggests that the AP-1 clathrin adaptor mediates HIV-1 envelope sorting from the TGN to the plasma membrane.[32] In simple retroviruses like MLV and SRV-3, a dileucine and a

tyrosine-based motifs in the viral envelope cytoplasmic tail have been shown to be critical for intracellular envelope trafficking.[33] Once the envelope glycoproteins reach the plasma membrane, they are incorporated into the virions. The remaining Env proteins are endocytosed and are recycled back to the plasma membrane for virus budding and release. Env proteins are incorporated into the virions in three ways: i) passive incorporation- without involving Gag and Env interactions ii) regulated incorporation that involves direct interactions between the Env and Gag (usually the matrix protein in case of HIV-1) and iii) regulated incorporation that involves interactions between Env and Gag through a linker protein.[28]

Apart from binding to receptors on the host cell and mediating fusion for virus entry, envelope glycoproteins also associate with receptor interference.[34] The retroviral Env usually blocks the receptor-binding domain or downmodulate cell-surface expression of the receptor such that the viral receptor on the cell surface is not available for re-infection by the same or other viruses. Because of this phenomenon, it is possible to group retroviruses into interference groups and infer the use of a common receptor, even when the receptors are unknown. Interference groups typically consist of closely related viruses, but can also comprise unrelated viruses that have converged on the same receptor. One such example is the RD114 and D-type interference supergroup which comprises of the type-D betaretroviruses and multiple exogenous and endogenous gammaretroviruses. All the viruses of the RDR interference supergroup use the sodium-dependent neutral amino acid transporter, ASCT2 as the receptor.[12]

The retroviral envelope glycoproteins are generally of two types- i) gamma-type and ii) beta-type.[11] In a gamma-type retroviral envelope, the SU and TM associate

covalently with each other by means of an intersubunit disulfide bond and has a characteristic immunosuppressive domain (ISD) (Figure 1.4).[11] The intersubunit disulfide bond formation is mediated by a CXnCC motif in the TM subunit. In contrast, the beta-type envelope is characterized by a non-covalent association between SU and TM and the absence of the ISD.[11] The gamma-type envelope is usually found in alpharetroviruses, gammaretroviruses and type-D betaretroviruses whereas the beta-type envelope is found in betaretroviruses and lentiviruses.[11] Since the viral envelopes determine host range, the acquisition of a gamma-type envelope by a virus has conferred some advantages over the beta-type envelope. For example, the gammaretroviruses have a very diverse host range and infect almost all vertebrates. On the other hand, the distribution of lentivirus is limited to mammals. This suggests that in contrast to the beta-type envelopes, the gamma-type envelopes may favor cross-species transmission between the vertebrates. As a result of the cross-species transmission, the gammaretroviruses probably infect a wide range of vertebrate hosts.

Evidence suggest that the gamma-type *env* gene has been swapped between different viral lineages by recombination on multiple occasions over a span of millions of years.[11] Such recombination events have given rise to several recombined viruses with diverse host range and tissue tropism. For instance, the viral lineage that gave rise to the type-D retroviruses such as the SRVs, originated as a result of the recombination between the *gag-pol* sequences of a beta-like virus and the *env* gene of a gamma-like virus. Hence, the type-D retroviruses share a high degree of sequence homology in their Gag and Pol proteins with the betaretroviruses, but their *env* gene resembles that of gammaretroviruses.[12] Some prominent examples of endogenous retroviruses with

gamma-type envelope include that of the Baboon endogenous retrovirus (BaEV), the feline RD114 and the Reticuloendotheliosis virus (REV) of chickens.[11] Reports suggest that the gamma-type envelopes of some endogenous retroviruses have also been exapted for host functions. For instance, the human *Syncytin-1* and the rabbit Syncytin-Ory*1* are fusogens that have been exapted for syncytiotrophoblast layer formation during placentation in mammals.[12,35] Thus gammaretroviral envelope glycoproteins form a powerful tool that helps to shed light on how the different phenomena such as host range, cross-species transmission and endogenization have impacted the evolution of retroviruses.[12]

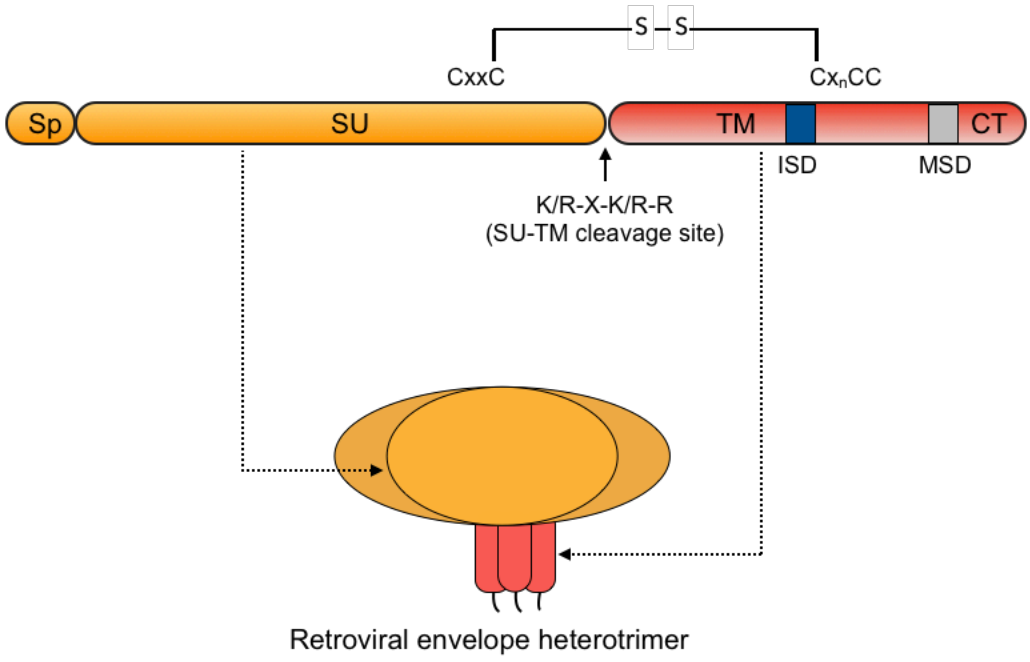


Figure 1.4. Schematic showing a typical gammaretroviral envelope glycoprotein. A typical gammaretroviral envelope glycoprotein has a surface subunit (SU; orange) with a hydrophobic signal peptide (Sp), a transmembrane subunit (TM; red), a membrane spanning domain (MSD; gray), an immunosuppressive domain (ISD; blue) and a cytoplasmic tail (CT). The conserved CxxC and CxnCC motifs form the intersubunit disulphide linkage between the SU and the TM subunits. The cellular protease cleaves the SU and the TM subunits at the K/R-X-K/R-R leading to the formation of the envelope heterotrimer.

1.5 Host restriction factors

Host restriction factors (RFs) may be defined as cellular factors that block different stages in the viral replication cycle either by directly interfering with the viral proteins or indirectly by rendering the host cellular milieu non-permissive for viral replication.[36] These RFs constitute a specialized form of innate immunity called the 'intrinsic immunity'. The RFs are mostly induced by interferons (IFNs), but sometimes they may also be constitutively expressed in certain cell types. The mechanism of antiviral activity of each RF is unique and is dependent on the extent of its viral targets. Therefore, an RF may either i) non-specifically restrict a broad range of unrelated viruses (e.g., tetherin), or ii) inhibit viruses in a species- or genus-specific manner (e.g., Fv4), or iii) inhibit viruses belonging to the same family (such as Trim-5α), or iv) inhibit unrelated viruses by targeting a common step in the viral replication cycle (e.g., APOBEC3G). Consequently, the viruses either encode dedicated antagonists or evolve their structural proteins to evade restriction by RFs. For example, HIV-1 counteracts Trim-5α by mutating its capsid protein while it encodes an accessory protein Vpu to counteract restriction by tetherin. The RFs have been engaged in a long-term 'evolutionary arms race' with their viral antagonists that leads to a series of adaptations and counteradaptations in the RFs and the viral antagonists respectively. The viral antagonists constantly exert a selection pressure on the RFs. As a result of the selection pressure, the RFs show signatures of positive selection which often form the target sites for the viral antagonists. Some RFs also act as pattern-recognition receptors (PRRs) and induce antiviral immune responses upon sensing viral infection.

The first RF called Fv1 was discovered in the early 1970s and was shown to inhibit MLV infection in mice.[37] Since then, there has been a growing list of RFs; especially the most well-characterized ones have been identified in the primate lentiviruses like HIV-1 and SIVs. Some notable examples of RFs that interfere with primate lentiviral infection are: The Interferon-induced transmembrane proteins (IFITMs), Tripartite motif 5-alpha (Trim-5α), APOlipoprotein B Editing Catalytic subunit-like 3 (APOBEC3) G, SAMHD1 (Sterile Alpha motif and Histidine-Aspartic domains containing protein 1), Mx2, and tetherin (Figure 1.5). The antiviral functions and the viral antagonists of the above-mentioned RFs are summarized in table 1.3.

Trim-5α recognizes and specifically binds to retroviral capsids and causes premature disassembly and degradation of the retroviral components to inhibit reverse transcription.[38] The Trim-5α is composed of the RING, B-Box, coiled-coil and a SPRY domain. The SPRY domain is thought to be the capsid recognition domain.[39] Apart from inhibiting primate lentiviral replication, new world monkey Trim-5α has been shown to inhibit SRV-3/MPMV infection.[40] However, SRV-3 is resistant to restriction by old world monkey Trim-5α.[40] As mentioned earlier in this section, the retroviruses usually counteract Trim-5α restriction by mutating their capsid proteins.

APOBEC3G is a cytidine deaminase that usually gets incorporated into the virions in the producer cells and restricts viral replication in the target cells. It causes deamination of cytidines to uracil in the minus strand of the viral DNA during reverse transcription.[38,41,42] As a result, the provirus acquires guanine to adenine mutations (often called hypermutations) which makes it incompetent for replication in the target cells.[38,42] There are several other members in the APOBEC3 family such as APOBEC3D,

APOBEC3F and APOBEC3H; all of the APOBEC3 proteins have been shown to suppress lentiviral replication. Consequently, the primate lentiviruses encode Vif (Viral infectivity factor) that prevents the incorporation of APOBEC3 proteins into the virions, by ubiquitination and proteosomal degradation of APOBEC3 proteins.[42]

SAMHD1 is a SAM- (Sterile Alpha motif) and HD- (Histidine-Aspartic) domains containing protein 1 that prevents reverse transcription of primate lentiviruses in the myeloid cells by depleting the dNTP pools required for the viral cDNA synthesis.[42,43,41] HIV-2 and some SIVs (such as the SIVrcm, SIVsmm and SIVmac) encode the accessory protein called Vpx (Viral protein x) which induces ubiquitination and proteosomal degradation of SAMHD1.[41,42] SIVagm encodes Vpr instead of Vpx that degrades SAMHD1.[44] HIV-1 lacks Vpx and is speculated to use the host cyclin L2 for degrading SAMHD1.[45]

RFs like Mx2, IFITM and tetherin have a broad spectrum of antiviral activity. IFITM proteins are entry blocks to viral replication and prevent fusion of the viral and host cell membranes. IFITMs are transmembrane proteins that multimerizes to form a network within the outer membrane leaflet and modulate the fluidity of the host cell membranes to prevent viral fusion. Unlike the other RFs, there are no known viral antagonists of IFITMs.

Mx2 (Human Myxovirus resistance 2) is a dynamin-like GTPase that inhibits the nuclear import and integration of the viral PIC by a mechanism that is independent of its GTPase activity and instead relies on its structural dimerization.[46,47] HIV-1 antagonizes Mx2 by introducing variations in its capsid protein.[48] Tetherin is the exit block to retroviral replication that restricts the egress of numerous enveloped viruses. The

structure and the detailed antiviral mechanism of tetherin are discussed in details in the section 1.5.1.

In general, restriction factor is a broad term and in addition to the well-characterized ones, there are several other host cellular proteins that interact with viruses to suppress virus replication. Some of them might fulfill one or more but not all criteria of a well-characterized RF. Reports suggest that most RFs are inefficient against the viruses in their native hosts but act as powerful barriers in the cross-species transmission events.[41]

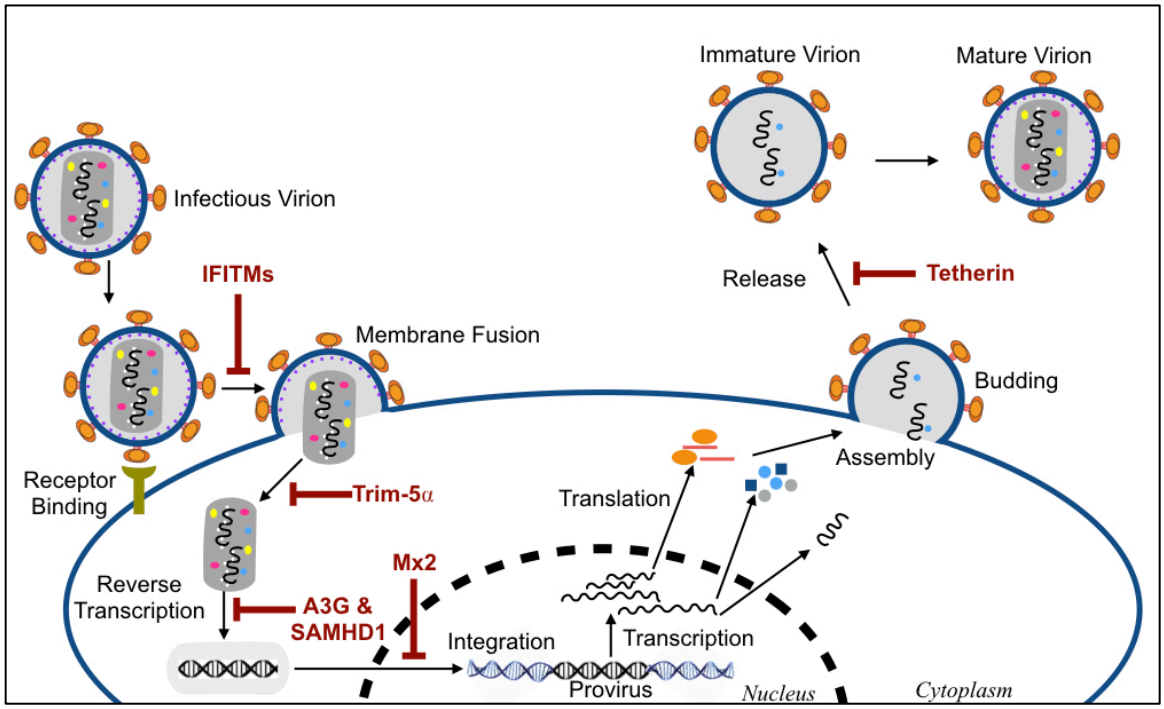


Figure 1.5. Schematic showing the blocks to replication imposed by some of the restriction factors. The different restriction factors are numbered and indicated by the red polygons. The first entry block is the membrane-spanning restriction factor called IFITM that prevents membrane fusion. Following entry, the uncoating of the retroviral capsid is inhibited by Trim-5α. APOBEC3G induces hypermutation and prevents reverse transcription of retroviral RNA in the target cells. SAMHD1 blocks reverse transcription by depleting the cellular dNTP pools. Mx2/MxB inhibits nuclear import of the pre-integration complex. Tetherin crosslinks enveloped virions to the plasma membrane and prevents the egress of virions from the surface of the infected cells.

Table 1.3. List of some well-characterized restriction factors.

Restriction Factor	Restriction inducible	Positive selection	Antiviral Function	Virus/es targeted	Viral antagonists
Trim-5α	Yes	Yes	interferes with the viral capsid uncoating	Retroviruses	Mutations in capsid
APOBEC3G	Yes	Yes	induces hypermutations in the provirus; inhibits reverse transcription in the target cells	Retroviruses & Hepadnaviruses	Vif (lentiviruses) GlycoGag (MLV) Bet (Spumavirus)
SAMHD1	Yes	Yes	depletes dNTP pools & inhibits the viral cDNA synthesis	Lentiviruses	Vpx (HIV-2 & some SIVs) Vpr (SIVagm)
IFITM	Some	Some	prevents fusion between the viral & the host cell membranes	Flaviviruses, Rhabdoviruses, Retroviruses, Bunyaviruses, Filoviruses, Reoviruses & Orthomyxoviruses	Unknown
Mx2/MxB	Yes	Yes	inhibits nuclear import of the PIC	primate lentiviruses & SRV-3	Mutations in capsid
Tetherin	Yes	Yes	restricts virion release	Flaviviruses, Rhabdoviruses, Retroviruses, Filoviruses, Paramyxovirus, Herpesvirus, Orthomyxovirus, Arenavirus & Togavirus	Unknown Unknown Vpu, Nef & Env Env (Ebola & Marburg virus) F & HN (Sendai virus) K5 (KSHV) & gM (HSV-1) NS1 & NA (Influenza virus) Unknown Nsp1(Chikungunya virus)

1.5.1 Tetherin- A potent block to enveloped viral release

Tetherin (also called HM1.24) is a specific cell surface biomarker found in terminally differentiated B-cells that was recognized after screening of mouse monoclonal antibodies raised against human plasma cells.[49] The tetherin gene was independently cloned from the synovial cells derived from human rheumatoid arthritis and was termed as BST2 (Bone marrow stromal cell antigen 2).[50,51] The protein was renamed as CD317 (cluster of differentiation 317) and was seen to be constitutively expressed at low levels in monocytes, macrophages, plasmacytoid dendritic cells and $CD34^+$ T-cells.[52] Tetherin is also widely expressed in salivary glands, gastrointestinal tract, pancreas, spleen, gall bladder and kidney tissues.[25]

In humans, the chromosome 19 contains a single tetherin gene.[50] However, ruminants like cows, sheep and goats have two copies of this gene due to gene duplication.[53,54] The tetherin promoter contains a single interferon regulatory factor (IRF) binding site and is strongly induced by IFN-α.[55] In some species and certain cell types, tetherin expression is also induced by IFN- β, -γ, -λ3, -τ and -ω.[56,53,57,58,59,60] Evidence suggest that in absence of IFNs, TLR 3, TLR 8 and IL-27 stimulate tetherin expression in the PBMCs.[55,61]

Tetherin has implications in the structural organization of cells. Tetherin aids in anchoring the apical actin network and in the stabilization and maintenance of Golgi network and membrane microdomain structures.[62,63,64,65] Tetherin also acts as a PRR and upon sensing viral infection induces antiviral response by triggering NF-κB activation.[66,67,68] Since both mouse and human-derived tetherin-specific monoclonal

antibodies had anti-tumor activity, several research groups proposed that tetherin can also be used as an immunotherapeutic agent against cancer.[69,70]

1.5.2 Discovery of tetherin as a host restriction factor

In certain cell lines like HELA, Jurkat T and Hep 2, HIV-1 accessory protein, Vpu was indispensable for HIV-1 viral release.[71,72,73] Hence, these cells were referred to as non-permissive cells. Contrary to these, HOS, COS, HEK-293T, HT-1080 and CV-1 cells were known as permissive cells as these cells permitted efficient HIV-1 particle release even in the absence of Vpu.[73,74,75] These observations suggested an absence of a host co-factor or the presence of a host restriction factor in the non-permissive cell lines. Further studies involving heterokaryons formed by permissive and non-permissive cells indicated that the non-permissive cells endogenously expressed a restriction factor which was counteracted by Vpu.[76,77] Thereafter several cellular factors like TASK-1 and CAML were thought to be the targets of Vpu.[78,79]

In 2008, the Bieniasz laboratory identified 'tetherin' as a host restriction factor.[80] They pointed out that Vpu expression in *trans* was necessary for the efficient release of HIV-1 virions in the non-permissive HeLa cells but not in HT1080, HEK-293T, Cos-7 and HOS cells. However, when HT1080, HEK-293T cells and HOS cells were induced by IFN-α, HIV-1ΔVpu virion release was inhibited. In contrast, when these IFN-α stimulated cells were treated with subtilisin protease, the trapped virions were released. Such an observation suggested the presence of a membrane-associated interferon-inducible antiviral protein in the non-permissive cell lines and the absence of the same in

the permissive cells.[75,80] This membrane-bound antiviral factor was termed as 'tetherin' and was recognized as a potent inhibitor of viral release.[80]

The Guatelli laboratory also made similar observations almost at the same time.[81] Using immunofluorescence microscopy, this group was able to visualize the co-localization of Vpu and tetherin within the endosomal compartments. The Gag proteins of both wild-type HIV-1 and HIV-1ΔVpu virus were also shown to co-localize with tetherin within the endosomes and at the plasma membrane.[81] Additionally, their research showed that Vpu downregulated tetherin from the cell surface.[81]

Interestingly, the antiviral function of tetherin was not only restricted to HIV-1 (retrovirus) but was also observed in case of Ebola virus (filovirus) release, suggesting that the restriction factor worked in a non-specific manner.[75]

1.5.3 Structure of tetherin

Tetherin is 181 amino acids long type II integral membrane protein that has a molecular mass ranging between 30 kDa to 38 kDa.[50] It is characterized by a small amino-terminal cytoplasmic tail (CT), an α-helical single-pass transmembrane domain (TM), an coiled-coil extracellular domain (EC) and a carboxy-terminal glycosyl-phosphatidylinositol (GPI) anchor (Figure 1.6A).[63] The C-terminus end of tetherin is cleaved off and replaced by the GPI anchor within the ER.[65] Tetherin localizes in the cholesterol-rich membrane microdomains through its GPI anchor. It should be noted that these lipid rafts also serve as preferable virus budding sites. Both CT and GPI anchors play a very important role in physically crosslinking the virions to the host cell membrane. Deletion of either of the membrane anchors inhibit the ability of tetherin to

block viral release.[82] Apart from localizing at the plasma membrane, tetherin is also found within TGN, early and recycling endosomes.

The tetherin CT contains a highly conserved non-canonical tyrosine motif (YxY) which favors recycling of tetherin between the cell membranes and the endosomal compartments. The YxY motif mediates endocytosis by binding to AP-1 and AP-2 clathrin adaptors.[57,62] There are two N-linked glycosylation sites on tetherin ectodomain (N_{65} and N_{92}), that are thought to have implications in the proper folding of tetherin and its anterograde transport.[82] Tetherin dimerizes by three cysteine residues (C_{53}, C_{63}, C_{91}) in its EC domain. This homodimerized extracellular domain confers conformational flexibility necessary for virion tethering.[82,83] Mutations in all three cysteine residues in the ectodomain inhibited tetherin dimerization and significantly reduced tetherin's ability to inhibit HIV-1ΔVpu viral release. However, mutations in the N-linked glycosylation sites had no such effects.[82,84,85] Reports suggest that tetherin ectodomains tetramerize through a 4-helix bundle interaction and this association seemed dispensable for tetherin's antiviral function.[82,83] Therefore, the significance of such a configuration remains obscure. However, one study speculated that the tetherin tetramers might be essential for proper trafficking of tetherin.[86]

In 2010, an X-ray crystallography study was conducted to solve the partial structure of tetherin ectodomain.[84] The authors revealed that the ectodomain of tetherin forms a 90 Å long rod-like, parallel, α-helical coiled-coil. Small angle X-ray scattering indicated that the N-terminal portion of this coiled-coil extending all the way up to the lumenal end of the TM domain is inclined at a slight angle.[84] There are structural irregularities within the coiled-coil, caused by the presence of some destabilizing residues

at the core of the α-helix. The formation of coiled-coil dimer restores this structural instability. The structural irregulaties maintain the flexibility of the ectodomain during tethering of the budding virions. The entire ectodomain is nearly 170 Å and loses its structural stability in the absence of the disulfide bonds.[84]

In the human tetherin, a leaky Kozak sequence is present before the methionine at position 13.[87] Therefore, this methionine also serves as an alternate start codon leading to the formation of a shorter tetherin isoform that lacks the YxY endocytosis motif and mostly localizes at the cell surface.[87] Shorter tetherin isoforms are also present in cats, elephants, horses and guinea pigs.[65,88]

The human tetherin orthologue lacks the five amino acids ($_{14}$GDIWK$_{18}$) in its cytoplasmic tail that are present in the other non-human primate tetherins. These five residues are also absent in Denisova and Neanderthal (the archaic humans), suggesting that the protective deletion arose roughly 800,000 years ago.[89]

1.5.4 Antiviral activity of tetherin

Tetherin dimer physically crosslinks budding enveloped virions to the host cell membrane and prevents their spread from one infected cell to another (Figure 1.6B). The tethered virions are internalized into the infected cells by endocytosis. A RING-type E3 ubiquitin ligase called Rabring7/BCA2 (Breast cancer-associated gene 2) interacts with tetherin and directs the trapped virions to the CD63-positive endosomes.[90] The tethered virions accumulate within the late endosomes and are finally degraded by lysosomal enzymes (Figure 1.6B).[90] The most unusual facet of tetherin is its non-specific mode of action. Virion tethering does not require recognition of any virus-specific molecular motif

but instead results from the anchoring of the N-and C- termini between the virion and cellular membranes. Therefore, in theory, tetherin can restrict the release of any entity that buds out of the cell; be it an enveloped virus or a membrane-bound vesicle.

A study conducted by Perez et al., have shown that an artificial tetherin (ART-tetherin) protein having identical structural domains (CT/TM, EC and GPI anchor) formed from three heterologous proteins exhibited a similar degree of restriction in the release of HIV-1ΔVpu and Ebola virus.[82] This finding indicates that the putative membrane topology and not the primary sequence homology to tetherin is necessary for tetherin's antiviral function.[82] The artificially engineered tetherin consisted of the transferrin receptor (TfR) TM domain, the coiled-coil domain of dystrophia myotonica protein kinase (DMPK) and the C-terminus GPI anchor of the urokinase plasminogen activator receptor (uPAR).[82] Similar to native tetherin, the deletion of either of the membrane anchors of ART-tetherin disrupted its antiviral function. However, HIV-1 Vpu was unable to counteract ART-tetherin and promote viral release.[82] This observation suggested that Vpu requires the recognition of specific residues within the native tetherin for its anti-tetherin activity.

Recently, Venkatesh and Bieniasz proposed that tetherin dimer acquires an 'axial configuration' and inserts either its N-termini pairs or C-termini pairs into the viral membranes during virion restriction.[91] Additionally, they noticed that there was a three- to a five-fold preference for the insertion of the GPI anchor into the viral membrane than into the host cellular membranes.[91] Inserting the N-termini in the host plasma membrane probably favors interaction with its cellular endocytosis machinery, and this could possibly explain the above preference.[91] A quantitative western blot analysis indicated

that a minimum of 12 tetherin dimers are required to cross-link a single virion to the cellular membrane.[91]

Tetherin has a broad spectrum of antiviral function against several families of enveloped viruses that includes retroviruses (e.g., alpha-, beta-, gamma-, delta-, lentivirus and spumaviruses),[92,93,94] paramyxovirus (Nipah virus),[95] rhabdovirus (VSV),[96] arenaviruses (Machupo virus and Lassa virus),[97,95] gamma-herpes virus (Kaposi's sarcoma-associated herpes virus; KSHV),[98] togavirus (Chikungunya virus), flavivirus (Dengue and Hepatitis C viruses)[99] and filoviruses like Ebola virus and Marburg virus.[100,92] Viruses like HSV-1 and HCoV-229E that bud from intracytoplasmic vesicular membranes are also restricted by tetherin.[101,102] The tetherin orthologue from the Gray-handed night monkey has a S164T mutation in its ectodomain and is the only tetherin orthologue lacking antiviral function.[103]

(A)

(B)

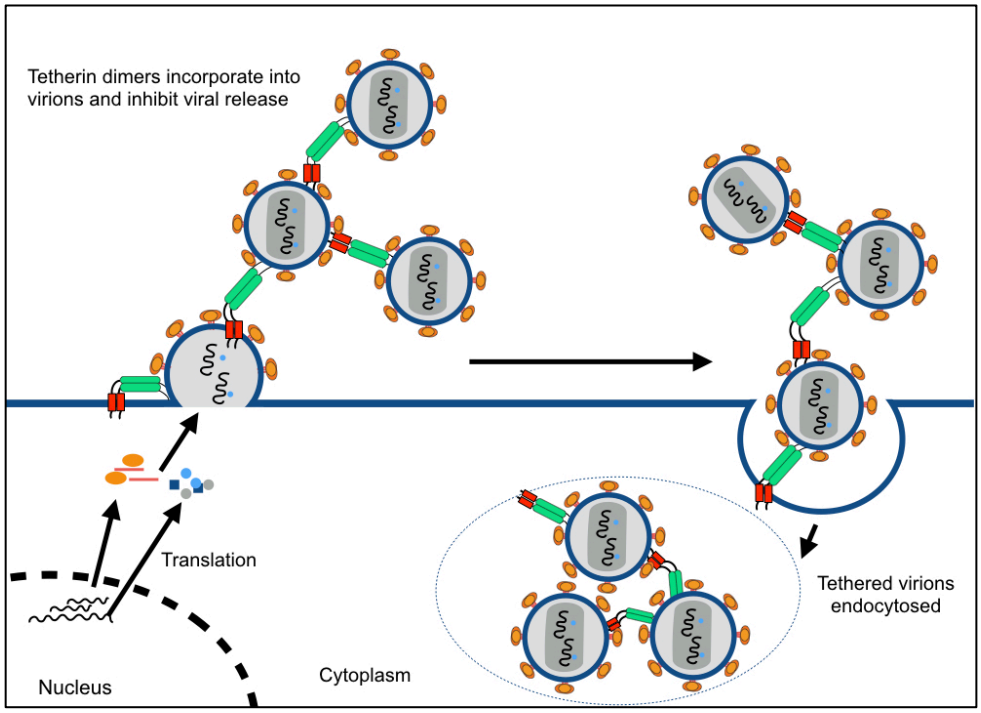


Figure 1.6. Tetherin and its antiviral activity. (A) Structure of a tetherin dimer. The different domains of tetherin are represented in this figure namely: CT= Cytoplasmic tail, TM= Transmembrane domain, EC= Ectodomain and GPI= glycophosphatidylinositol anchor. Cysteine residues on the EC forms disulfide bridges and help in the dimerization of tetherin molecules. Dimerization is imperative for its antiviral function. (B) Antiviral action of tetherin. Tetherin dimers tether virions to the host cell membrane and restrict their egress. Tetherin traps viral particles by targeting their host cell-derive lipid envelope. The tethered virions accumulate within the early and late endosomes and are finally degraded within the lysosomes.

1.5.5 Role of tetherin in promoting viral pathogenesis

Retroviruses mostly disseminate within the host by cell-free transmission in which the viruses are released into the extracellular milieu.[104,105] However, sometimes the cell-free virus transmission may become unfeasible. Under such circumstances, the viruses prefer to disseminate via direct cell-cell contacts, and this is facilitated by the formation of virological synapses.[104,105,106] The formation of virological synapse involves rearrangement of the cytoskeleton, and interactions between the viral envelope glycoprotein on the donor cell and the receptor on the target cell. Although tetherin is well-known for inhibiting cell-free virus transmission, some researchers believe that tetherin allows cell-cell virus transmission.[107,108,109] To investigate the role of tetherin in the cell-cell transmission of HIV-1, Jolly et al., performed a flow-cytometry based quantitative cell-cell transmission assay. They noticed that tetherin accumulated at the virological synapse (VS) and increased VS formation in HIV-1ΔVpu infected CD4+ T-cells compared to the wild-type HIV-1 infected CD4+ T-cells.[108] In contrast, siRNA-mediated knockdown of endogenous tetherin expression reduced VS formation and cell-cell dissemination of both wild-type and mutant HIV-1 indicating that tetherin may be critical for cell-cell viral spread.[108] However, this functional attribute of tetherin is still debatable and needs further investigation.

1.6 Viral antagonists of tetherin

As mentioned earlier, tetherin has a non-specific mode of action and does not require the recognition of any viral proteins for restriction. Therefore, the viruses cannot counteract tetherin by acquiring escape mutations in their structural proteins. Different enveloped viruses have evolved various tetherin-evasion mechanisms. Some viruses actively evade tetherin-mediated restriction by encoding dedicated tetherin antagonists, while others (like HIV-2 and Ebola) use their envelope glycoproteins as tetherin antagonists (Figure 1.7). In both the cases, the tetherin antagonists either sequester tetherin within the endosomal compartments or prevent anterograde transport or recycling of tetherin. Some other viruses exclude tetherin and bud from membrane domains lacking tetherin, or spread by direct cell-cell contact. Lastly, certain viruses inhibit the interferon pathway such that the sensors of viral infections do not trigger tetherin expression.[110,111] The different viral strategies for tetherin-antagonism are discussed in the following sections.

1.6.1 Viral accessory proteins as tetherin antagonists

Among the primate lentiviruses, SIVs of Old world monkeys lack a functional Vpu gene and encode an accessory protein called Nef (negative regulatory factor) to overcome restriction by their respective simian host tetherins.[112,113,114,115] These include the Nef proteins of SIVs from rhesus macaques, sooty mangabey, pig-tailed macaques, blue monkey, African-green monkey and chimpanzees. Nef is a 27-35kDa myristoylated cytosolic protein and is well-known for modulating the expression of numerous membrane proteins.[116,117] The Nef protein is characterized by a globular core domain, an

N-terminus region and a C-terminus flexible loop.[118] The di-acidic residues ($_{155}$EE$_{156}$) and an acidic patch ($_{62}$EEEE$_{65}$) in the globular domain interacts with COP proteins (β-COP) and PACS1 and -2 proteins (phosphofurin acid cluster sorting proteins) respectively.[119,120,121] The C-terminus loop of Nef contains a $_{160}$D/ExxxLL$_{165}$ motif that recruits the clathrin adaptors. Thus, although the general function of Nef is to link the vesicular machinery with its protein targets for endocytosis, this general function also forms the basis of tetherin-antagonism in Nef. SIV Nef protein not only downregulates the surface expression of CD4, CD28, MHC class I and class II, it is also reputed for antagonizing tetherin by downmodulating non-human primate tetherin levels from the cell surface.[112,114,116] The specificity of the Nef protein of SIVmac (SIV of rhesus macaque) for rhesus tetherin mapped to a five amino acid motif ($_{14}$G/DDIWK$_{18}$) in the rhesus tetherin cytoplasmic tail that are absent from the human tetherin orthologue.[112] Thus, SIVmac Nef is non-functional against human tetherin and fails to downregulate cell surface expression of human tetherin. The anti-tetherin activity of SIV Nef is usually species-specific and no studies have been conducted till date to determine if it can counteract other mammalian tetherin orthologues.

The detailed mechanism of SIVmac Nef's anti-tetherin function was elucidated by the Evans laboratory in 2013.[122] They pointed out that the mechanism of tetherin evasion in Nef was dependent on clathrin-mediated endocytosis. The researchers reported that highly conserved $_{160}$D/ExxxLL$_{165}$ motif in the C-terminus loop of Nef protein is necessary for Nef's anti-tetherin activity.[123,122,124] Mutations within this motif impaired Nef's ability to downregulate cell surface tetherin expression.[122,124] Using alanine scanning mutagenesis, Serra-Moreno et al., identified a number of residues within the

globular core (27 residues), N-terminus (9 residues) and C-terminus (7 residues) of Nef that were imperative for Nef-mediated tetherin-antagonism.[122] This suggests that the anti-tetherin function of Nef is not dependent on a single structural domain but results from the interactions among all the Nef domains. Nef was also shown to directly associate with the rhesus tetherin cytoplasmic tail and remove rhesus tetherin from viral budding sites.[122]

Kluge et al., revealed that the Nef protein of HIV-1 O group have also evolved to antagonize human tetherin.[125] The domain of interaction between HIV-1 O Nef and human tetherin mapped to the residues $_{5}$SYDY$_{8}$, which lie in close proximity to $_{14}$G/DIWK$_{18}$ motif.[125] Mutations in $_{5}$SYDY$_{8}$ residues disrupted Nef's ability to reduce cell surface human tetherin levels. HIV-1 O Nef also sequestered newly formed human tetherin within the TGN and prevented their anterograde transport to the plasma membrane.[125] Recently, it has been reported that the HIV-1 group O strain RBF206 Vpu counteracts both long and short isoforms of human tetherin in a non-specific manner.[126]

Unlike most SIVs, HIV-1 groups M and N antagonize the antiviral effect of human tetherin by encoding another accessory protein called Vpu (Viral protein U).[80,112,93,127,113] The Vpu proteins of the immediate predecessors of HIV-1, SIVcpz and SIVgor lost their ability to antagonize tetherin.[113,128] Hence, both SIVcpz and SIVgor encode Nef to evade restriction by tetherin.[113,128] In contrast, the Vpu proteins of SIVs from greater spot-nose monkey, mustached monkey, Dent's mona monkey and Mona monkey are able to antagonize their respective host tetherins.[113,128] The HIV-1 group M Vpu specifically antagonizes human tetherin but is sensitive to non-human primate tetherins.[129,130] Vpu is a small transmembrane protein that has a very short luminal

region, a 23 amino acids long α-helical transmembrane domain and a cytoplasmic tail consisting of two α-helices and a flexible hinge. Vpu interacts with the TM domain of human tetherin to evade restriction by human tetherin.[131,132] The residues A_{14}, A_{18} and W_{22} in the transmembrane domain of Vpu are critical for tetherin-antagonism.[133] Although the exact role of these residues in tetherin-antagonism is still dubious. The specificity of Vpu for human tetherin maps to the residues I34, L37, L41 and T45 in the transmembrane domain of human tetherin.[134]

The mechanism of tetherin-antagonism by HIV-1 group M Vpu protein involves ubiquitination and proteosomal degradation of human tetherin.[135] Vpu has a highly conserved $_{51}$DSGxxS$_{56}$ motif present in between the two α-helices and undergoes phosphorylation by casein kinase-II enzyme. This phosphorylation event favors the recruitment of the β-transducing repeat-containing protein (βTrCP) subunits of Skp1-Cullin1-F-box ubiquitin ligase, which results in ubiquination followed by proteosomal degradation.[136] However, the exact cytoplasmic tail residues of human tetherin that are targeted for ubiquitination have not yet been identified.[135] It should be mentioned in this regard that the shorter isoform of human tetherin is relatively resistant to Vpu suggesting that some or all of the first twelve residues in the human tetherin CT might be crucial for ubiquitination.[87] The loss of interaction between βTrCP and the Vpu cytoplasmic domain renders Vpu sensitive to human tetherin.[137,138,139]

Two independent studies have proposed that Vpu prevents the anterograde transport of newly synthesized tetherins and inhibits recycling of tetherin between the TGN and the cell surface.[140,141] The $_{59}$ExxxLV$_{64}$ motif in the second α-helix of the Vpu CT impairs tetherin trafficking, and removes human tetherin from viral assembly

sites.[132,142] The $_{59}$ExxxLV$_{64}$ motif of Vpu also interacts with AP-1 clathrin adaptor and causes internalization of Vpu-tetherin complex within the endosomal compartments.[143,144] Thereafter, the Vpu-tetherin complex undergoes ubiquitination and lysosomal degradation. The phosphorylation of S_{52} and S_{56} residues has also been shown to be critical for the recruitment of AP-1 and AP-2 clathrin adaptor proteins suggesting that the phosphorylation step leads to two independent events: i) βTrCP-dependent ubiquitination and ii) clathrin-mediated endocytosis.[145] The cytoplasmic tail of HIV-1 group N Vpu protein also contains a similar $_{59}$DxxxLV$_{64}$ motif and potently antagonizes human tetherin.[146] Interestingly, a recent report suggested that some alleles of HIV-1 group M Nef proteins are also able to evade tetherin-mediated restriction by downregulating human tetherin from the cell surface.[147] Arias et al., showed that the longer tetherin isoform and not the shorter isoform was sensitive to group M Nef. HIV-1 M Nef was shown to physically interact with human tetherin by targeting the first 12 residues in the human tetherin CT that are absent in the shorter isoform.[147] This research elucidates that in absence of a functional Vpu protein, HIV-1 group M Nef proteins may adapt to evade tetherin-mediated restriction.[147]

The Kaposi's sarcoma herpes virus (KSHV) encodes a RING-CH E3 ubiquitin ligase protein called K5 that antagonizes human tetherin.[98,148,149] K5 targets the lysine residues at position 18 and 21 in the cytoplasmic tail of human tetherin for ubiquitination of tetherin.[98] Glycosylation of tetherin is necessary for the anti-tetherin activity of K5 suggesting that the interaction between tetherin and K5 takes place within vesicular compartments outside the ER.[149]

Studies have shown that the non-structural proteins of Chikungunya virus (Nsp1) and Influenza virus (NS1) promote virion release in the presence of tetherin.[137,150,151] Additionally, the Influenza A virus neuraminidase proteins are also known to counteract tetherin by an undefined mechanism.[152]

1.6.2 Viral envelope glycoproteins as tetherin antagonists

HIV-2 arose as a result of independent cross-species transmission of SIV from sooty mangabey (SIVsmm) into humans, and unlike HIV-1, HIV-2 lacks a Vpu gene. Previous studies indicated that the envelope (Env) glycoprotein of HIV-2 had Vpu-like activity, and enhanced HIV-1ΔVpu particle release in the non-permissive cell lines.[153] In 2009, Tortorec et al., demonstrated that HIV-2 group A envelope potently antagonized human tetherin, and this Env-mediated tetherin-antagonism was dependent on the proteolytic cleavage of the envelope into its respective glycoprotein subunits (gp120 and gp41).[93] HIV-2 Env reduced cell surface human tetherin levels without affecting the total cellular tetherin levels.[93,154] A conserved tyrosine-based endocytosis motif (GYXXφ) in the gp41 subunit of the HIV-2 Env was shown to be critical for this function.[93] The interaction between HIV-2 Env and human tetherin mapped to the ectodomain of both the proteins, and this interaction was necessary but not sufficient for tetherin-antagonism by HIV-2 Env.[93,155] Additionally, the alanine residues at position 97, 100, 104 and 107 in the human tetherin ectodomain rendered human tetherin sensitive to HIV-2 Env.[155] A more recent study indicated that the asparagine residue at position 659 (N_{659}) in the HIV-2 Env TM subunit is indispensable for its tetherin-antagonism.[156] Mutation of this residue to an aspartate impaired the ability of HIV-2 Env to counteract human tetherin.[156] HIV-2 Env also sequestered tetherin within the TGN without causing its degradation.[93,154]

The discovery of the tetherin-antagonism in HIV-2 Env was indeed very significant as it was the first evidence of tetherin-antagonism in a retroviral envelope glycoprotein. Thereafter, several independent research groups started hunting for tetherin-antagonism in other retroviral envelope. Recently, Heusinger et al., proposed that the envelope glycoproteins of some isolates of SIV from sooty mangabey (SIVsmm) exhibit anti-tetherin activity.[157] They proposed that since SIVsmm has adapted both the Nef and the envelope glycoprotein to counteract tetherin; and this possibly accounts for the successful cross-species transmission of SIVsmm into humans on nine different occasions.[157]

The presence of tetherin-antagonism in a simian retroviral envelope was first demonstrated by the Towers laboratory in 2009.[158] They found out that in the absence of a functional Nef protein, the Env of SIV from tantalus monkey (SIVtan; a distant relative of HIV-1) was able to counteract human as well as some non-human primate tetherin orthologues.[158] Like HIV-2 Env, the SIVtan Env also downregulated cell surface human tetherin levels and the specificity of the Env for tetherin mapped to the alanine residue at position 100 in the ectodomain of human tetherin.[158]

In 2011 the Evans laboratory demonstrated that the envelope glycoprotein of a pathogenic *nef*-deleted SIV of rhesus macaques (SIVmacΔ*nefP*) acquired the ability to antagonize macaque tetherin after serial passage in the host.[159] This tetherin-adaptive viral envelope was referred to as EnvITM, where ITM stands for *improved transmembrane*. The ITM was previously shown to restore pathogenicity of SIVmacΔ*nefP* in rhesus macaques.[160] Serra-Moreno et al., showed that the EnvITM physically associates with rhesus tetherin and downmodulates rhesus tetherin from the

cell surface.[159] Grafting of the EnvITM cytoplasmic tail onto a heterologous protein restored resistance to tetherin suggesting that the EnvITM CT was sufficient for counteracting tetherin. The tyrosine residue at position 721 in the EnvITM was identified as the viral determinant of tetherin-antagonism. Unlike SIVmac Nef, the target of EnvITM overlapped a stretch of ten amino acids in the rhesus tetherin CT.[159]

This research raises the possibility that several such naturally occurring SIVs might also be able to adapt their envelope glycoproteins to antagonize their respective host tetherins in the absence of a dedicated tetherin antagonist (such as SIV Nef or HIV-1 Vpu). However, in reality adapting a viral envelope glycoprotein for tetherin-antagonism might have its consequences on viral fitness. For instance, since both HIV-2 Env and EnvITM sequester tetherin within the endosomal compartments, this might lead to a reduction in the anterograde trafficking of the Env proteins and a consequent decrease in the infectious viral titer. In order words, this might lead to a reduction in viral pathogenicity.

The envelope glycoprotein of feline immunodeficiency virus (FIV) has also been shown to antagonize carnivore tetherins by an unknown mechanism that does not require the downregulation or degradation of tetherin but instead results from the efficient incorporation of the envelope glycoprotein into the FIV particles.[88,161] FIV Env expression in *trans* rescues *env*-defective FIV particles but not Vpu-deleted HIV-1 from tetherin suggesting that the anti-tetherin function of FIV Env is specific to FIV.[161] Western blot analysis revealed that there was an enhancement in the wild-type FIV particle production compared to the *env*-defective mutant FIV particles in the presence of tetherin.[161] This observation led the investigators to hypothesize that tetherin might be

functioning as a co-factor rather than a restriction factor during FIV budding and release.[161]

Yin et al., reported that envelope glycoprotein of the equine infectious anemia virus (EIAV) antagonized tetherin in a species-specific manner.[162] They observed that the transient expression of EIAV Env was sufficient to rescue *env*-deleted EIAV from equine tetherin but not from human tetherin.[162] Additionally, data from the co-immunoprecipitation assay indicated that EIAV Env physically interacts with equine tetherin but not with human tetherin.[162] Interestingly, the EIAV Env expression did affect the cellular distribution of equine tetherin but it did not degrade equine tetherin.[162] It might be possible that the EIAV Env sequesters tetherin within the intracellular compartments thereby prevents the recycling of tetherin as well as anterograde transport of *de novo* synthesized tetherins. However, this research needs further experimental validation because it is possible that the transient expressions of tetherin and the viral envelope constructs may have impacted the experimental results.

By far, most of the exogenous retroviral envelope glycoproteins have been characterized as tetherin antagonists. To date, there has been single evidence that suggests that an endogenous retroviral envelope glycoprotein might also function as a tetherin antagonist. A study conducted by the Dewannieux laboratory showed that the HERV-K Env protein has anti-tetherin function against human and OWM tetherins.[163] Co-immunoprecipitation experiments indicated a possible physical interaction between the SU subunit of the HERV-K Env and human tetherin. However, this interaction alone was not sufficient for efficient tetherin-antagonism.[163] Similar to EIAV and FIV Envs, the HERV-K Env does not downmodulate or degrade tetherin.[163] Therefore, unlike the

primate lentiviral tetherin antagonists, HERV-K Env lacks a distinct tetherin-evasion mechanism and requires further investigations.

The first evidence for Env-mediated tetherin-antagonism in a filovirus envelope glycoprotein was reported by the Bates laboratory in 2009.[100] The investigators reported that the Ebola virus glycoprotein (GP) exhibited tetherin-antagonism against human, murine and short isoform of feline tetherin.[100,161] Ebola GP also antagonized artificially engineered tetherin (ART-tetherin) and promoted virion release.[100,164] This suggest that the anti-tetherin activity of Ebola GP does not require the recognition of any specific sequence/s in tetherin. The transient expression of Ebola GP also rescued a heterologous virus from tetherin.[100] A recent study proposed that the membrane spanning domain (MSD) of the GP2 subunit and the glycan cap of the GP1 subunit of Ebola glycoprotein are critical for tetherin-antagonism.[165] However, the Ebola GP neither downmodulates cell surface expression of tetherin nor degrades it.[164] Unlike HIV-2 Env, tetherin-antagonism is also not dependent on the proteolytic cleavage of the viral glycoprotein.[100] Therefore, additional studies are required to- decipher the tetherin-evasion mechanism in Ebola GP and identify the tetherin domains targeted by the Ebola GP for tetherin-antagonism.

Unlike KSHV, the herpes simplex virus-1 and -2 (HSV-1 and HSV-2) do not encode any accessory protein for tetherin-antagonism. Reports suggest that these viruses use their envelope glycoprotein to evade restriction by human tetherin.[101,166] The envelope glycoprotein gM of HSV-1 exhibits moderate levels of anti-tetherin against human tetherin.[101] Another study pointed out that HSV-1 may also promote viral release by encoding the virion host shut-off protein (Vhs) which depletes the tetherin mRNA

transcripts.[167] In contrast, multiple envelope glycoproteins of HSV-2 (such as gB, gD, gH and gL) actively antagonized human tetherin by downregulating cell surface human tetherin levels.[166] The envelope glycoproteins of Sendai virus (paramyxovirus): the fusion protein (F) and the haemagglutinin-neuraminidase protein (HN) have been reported to degrade tetherin to evade tetherin-mediated restriction.[168]

The different viral antagonists of tetherin and their tetherin-evasion mechanisms are summarized in Table 1.4.

Table 1.4. List of some well-characterized tetherin antagonists and their mechanism of tetherin-evasion.

Virus	Family	Antagonist	Species-specificity	Tetherin domain targeted	Mechanism of tetherin-antagonism
HIV-1 Group M & N	*Retroviridae*	Vpu	Human, gorilla & Chimpanzee	TM	Downregulation
HIV-1 Group O	*Retroviridae*	Nef	Human	CT	Downregulation
HIV-2	*Retroviridae*	Envelope	Human	EC	Downregulation
SIVgsn, SIVmus, SIVmon & SIVden	*Retroviridae*	Vpu	Simian	TM	Downregulation
SIVcpz, SIVgor, SIVmac, SIVsyk, SIVrcm, SIVsmm, SIVagm	*Retroviridae*	Nef	Simian	CT	Downregulation
SIVmacΔ*nefP*	*Retroviridae*	EnvITM	Rhesus Maaque	CT	Downregulation
SIVtan	*Retroviridae*	Envelope	Human & OWMs	EC	Downregulation
FIV	*Retroviridae*	Envelope	Canine & Feline	Unknown	Unknown; requires Env incorporation into virions
EIAV	*Retroviridae*	Envelope	Equine	Unknown	Unknown
HERV-K	*Retroviridae*	Envelope	Human & OWMs	Undetermined	Unknown
Ebola	*Filoviridae*	Ebola GP	Human, mouse & ART-tetherin	Unknown	Unknown
KSHV	*Herpesviridae*	K5	Human	CT	Downregulation
HSV-1	*Herpesviridae*	gM	Human	Unknown	Unknown
HSV-2	*Herpesviridae*	gB, gD, gH & gL	Human	Unknown	Downregulation
Chikungunya virus	*Togaviridae*	Nsp1	Human	Unknown	Downregulation
Influenza virus	*Orthomyxoviridae*	NS1 & NA	Human	Unknown	Unknown; impedes tetherin induction
Sendai virus	*Paramyxoviridae*	F/HN	Human	Unknown	Degradaes tetherin by an unknown mechanism

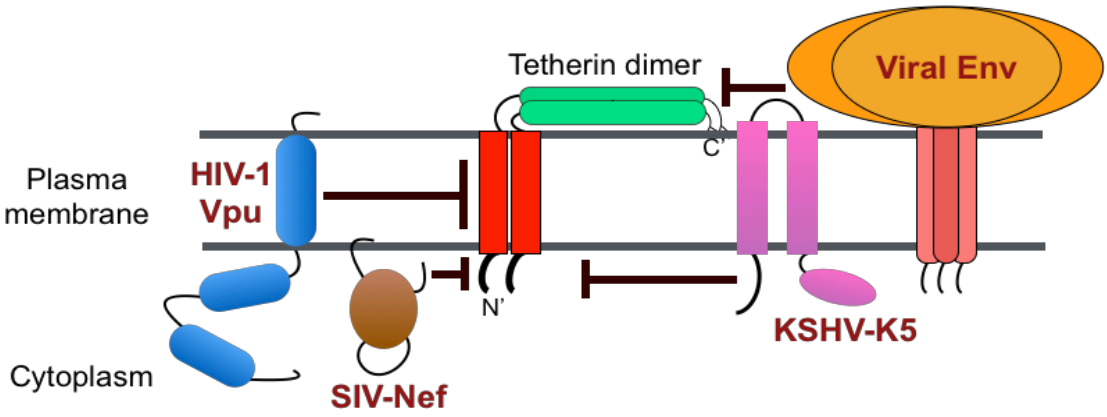


Figure 1.7. Viral antagonists of tetherin and their domains of interaction. HIV-1 encodes an accessory protein Vpu (blue) that antagonizes human tetherin by targeting the transmembrane domain of human tetherin. SIVs encode another accessory protein called the Nef (brown) that antagonizes their respective simian host tetherin by targeting the tetherin cytosolic domain. Kaposi's sarcoma herpes virus (KSHV) encodes K5 (pink) protein that ubiquitinates human tetherin by targeting the lysine residue at position 18 in the cytoplasmic tail of human tetherin. Viruses like Ebola and HIV-2 use their envelope glycoproteins (the SU subunit shown in orange and the TM subunit shown in red) as tetherin antagonists.

1.7 Concluding remarks

To date tetherin-evasion mechanisms have been extensively studied in complex retroviruses like HIV-1 and SIV that encode Vpu and Nef respectively to counteract restriction by their respective host tetherins.[80,112] The envelope glycoproteins of Ebola virus, HSV-1 and HERV-K have been shown to function as tetherin antagonists but their mechanism of tetherin-antagonism is not well-defined. The mechanism by which numerous simple retroviruses (such as the alpharetroviruses, betaretroviruses and gammaretroviruses) evade restriction by tetherin is still unknown. Therefore, my research focused on determining whether simple retroviruses evade restriction by their host tetherins, and if so, on delineating the mechanism of evasion. This research stemmed from the observation that SRV-3, a simple type-D retrovirus was sensitive to restriction by human tetherin [92]. However, given that the natural host of SRV-3 is the rhesus macaque and together with the published reports that rhesus macaque tetherin is functional against other retroviruses both in vitro and in vivo,[112] I hypothesized that SRV-3 must be resistant to restriction by rhesus macaque tetherin. I have identified the SRV-3 envelope glycoprotein as a potent tetherin antagonist and have also expanded the research to simple retroviruses outside the betaretrovirus genus. This research has finally opened the door to the discovery that tetherin-antagonism may be a novel and conserved function of many gammaretroviral envelopes.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plasmids

2.1.1 Proviral plasmids

The full-length SRV-3 proviral plasmid (pSARM4) was a gift from Dr. Eric Hunter, Emory Centre for AIDS Research at the Emory University, Atlanta, GA. Song et al., has previously described the construction of these plasmids.[169] In the pSARM-eGFP proviral plasmid, the functional *env* gene was replaced with an EGFP.[170] This plasmid was used in the single-cycle virus release assays to produce the SRV-3Δ*env*. The SIVmac239-VIEGFP plasmid (obtained initially from the Hung Fan laboratory at the University of California, Irvine, CA) was modified to express the Gag and Pol proteins.[171] The SIVmac239-VIEGFP plasmid was used for transfections in the single-cycle VLP release assay to produce SIV particles, which lack both functional *nef* and *env* genes; these viral particles are referred to as SIVmac239Δ*nef*Δ*env* virus in this dissertation.

2.1.2 Tetherin plasmids

The pCDNA3.1-based tetherin orthologues from human, rhesus macaque, sooty mangabey and pig-tailed macaque as well as human-rhesus tetherin reciprocal chimeras and cytoplasmic tail mutants were kindly provided by Dr. Ruth Serra-Moreno, Texas Tech University, Lubbock, TX. The detailed strategies of cloning these tetherin expression constructs have been previously described by the Evans laboratory.[112] Using the appropriate species-specific tetherin-HA forward and tetherin-HA reverse primers, an ectodomain-HA epitope was added to the ectodomain of all the tetherin expression constructs by site-directed mutagenesis. The HA-epitope was inserted after residue 131 in human tetherin and after residue 134 in case of all the OWM tetherins. All of these HA-tagged tetherin orthologues were amplified by PCR using their respective species-specific

tetherin-AgeI forward and tetherin-BamHI reverse primers. The newly amplified HA-tetherin orthologues were subcloned into a retroviral packaging vector, pQCXIP (having the puromycin-resistance marker) using AgeI and BamHI as restriction sites. HA-tagged tetherin orthologues from baboon (Accession XP_003915187), squirrel monkey (Accession XP_003942316), cat (Accession NP_001230014) and dog (Accession XP_865603) were synthesized by ThermoFisher Scientific GeneArt Gene Synthesis and Services. These constructs were also amplified by PCR using their respective species-specific tetherin-AgeI forward and tetherin-BamHI reverse primers and then subcloned into pQCXIP. In order to clone the African-green monkey (AGM) tetherin orthologue, RNA was isolated from Vero cells using Trizol reagent (Ambion/Life Technologies). Two-step RT-PCR was done to procure the AGM-tetherin cDNA by using Transcriptor First Strand cDNA Kit (Roche) and anchored-oligo$(dT)_{18}$ primer. The AGM-tetherin cDNA was amplified using AGM-tetherin AgeI forward and BamHI reverse primers and subcloned into pQCXIP. After cloning the AGM-tetherin, the HA-epitope was inserted in its ectodomain by site-directed mutagenesis. The HA-rhesus tetherin cytoplasmic tail point mutants in pCGCG were also generated by site-directed mutagenesis. All the primers used for PCR amplification and HA-tagging of the tetherin constructs are listed in table 2.1.

2.1.3 Nef and Vpu plasmids

Both SIVmac239-Nef and HIV-1 NL4-3 Vpu (gifts from Dr. Ruth Serra-Moreno, Texas Tech University, Lubbock, TX) were subcloned into a bicistronic expression vector pCGCG using the XbaI and MluI as restriction sites. The pCGCG bicistronic construct is transcriptionally linked to GFP via an IRES.[112]

2.1.4 Viral envelope plasmids

The envelope genes from squirrel monkey retrovirus (SMRV; Accession M23385), feline RD114 virus (Accession AB705392), Baboon endogenous retrovirus (BaEV; Accession AB979448.1), Reticuloendotheliosis virus (REV; Accession AAW57301) and simian retrovirus serotypes 1 (Accession M11841), 2 Accession M16605), 4 (Accession ADC33202) and 5/Y (Accession AB611707) were commercially synthesized by ThermoFisher Scientific GeneArt Gene Synthesis and Services. All these viral envelopes were amplified by PCR and subcloned into the pCGCG vector using XbaI and MluI sites. The SRV-3 envelope expression plasmid (pTMO) was generously provided by Dr. Eric Hunter, Emory Centre for AIDS Research at the Emory University, Atlanta, GA.[172] The SRV-3 *env* was amplified using SRV-3 *env*-specific XbaI forward and MluI reverse primers and subsequently cloned into pCGCG vector. A tagged version of SRV-3 *env* in pCGCG was also generated by adding a C-terminus Avi epitope using site-directed mutagenesis. The untagged SRV-3 *env*-pCGCG plasmid was used for both single-cycle VLP release assay and downregulation assay. The Avi-tagged SRV-3 *env*-pCGCG plasmid was used for the co-immunoprecipitation experiment. The pCGCG-SRV-3 *env* trafficking mutants Y23S, Y35S and L3S/Y23S were generated using round-the-horn-PCR. The SRV-3 *env* cytoplasmic tail alanine scanning mutants and the trafficking mutant L3S were also commercially synthesized by ThermoFisher Scientific GeneArt Gene Synthesis and Services. All of these fragments were subcloned into a truncated SRV-3 *env*-pCGCG vector using PstI and MluI sites. The truncated SRV-3 *env*-pCGCG vector consists of the entire pCGCG vector, the full-length SRV-3 *env* SU domain and the partial extracellular domain of the SRV-3 *env* TM subunit before the PstI site. The

plasmids expressing the Vesicular Stomatitis virus envelope glycoprotein, pVSV-G and the ecotropic Murine Leukemia virus envelope glycoprotein was obtained from the Clonetech Laboratories. All the primers used for PCR amplification and Avi-tagging of the viral envelope constructs are listed in tables 2.2.

2.2 Maintenance of cell-lines

The Human Embryonic Kidney cells 293T/17 (HEK-293T/17), GP2-293 cells, Vero cells and HT1080 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin-Streptomycin solution and 1% L-Glutamine. All HEK-293T based stable cell-lines expressing HA-tagged tetherin orthologues were maintained in DMEM/10% FBS containing 4 μg/ml of puromycin. All cell lines were maintained in 37^{o} C incubators with 5% CO_2.

2.3 Generation of stable cell lines

GP2-293 cells were seeded at a density of 5x10^5 cells per well in 6-well plates for transfection. GP2-293 cells are HEK based retroviral packaging cells stably expressing the retroviral *gag* and *pol* genes. Using the GenJet lipid-based transfection system, these cells were transiently cotransfected using 15 μg of pVSV-G and 15 μg of an empty vector, pQCXIP only or pQCXIP-HA-tagged tetherin orthologues or rhesus-human tetherin reciprocal chimeras/mutants or rhesus tetherin cytoplasmic tail mutants to produce VSV-G pseudotyped retroviral particles packaging these vectors. Forty-eight hours post-transfection, one milliliter of the infectious cell culture supernatants containing VSV-G pseudotyped retroviral particles packaging either pQCXIP vector only

or HA-tetherin-pQCXIP were used to transduce HEK-293T/17 cells. The HEK-293T/17 cells were seeded at a density of $5x10^5$ cells per well in 6-well plates twenty-four hours before transduction. Forty-eight hours post-transduction, the stable cell lines were selected in DMEM/10% FBS supplemented with 10μg/ml of puromycin and eventually grown in DMEM/10% FBS containing 4 μg/ml of puromycin. Stable 293T cell-lines expressing rhesus and human tetherin orthologues were gifted by Dr. David T. Evans, University of Wisconsin-Madison, Madison, WI.[112]

2.4 Single-cycle virus-like-particle release (VLPs) assays

2.4.1 By transient transfections

All transfections were performed in 6-well plates. $5x10^5$ HEK-293T/17 cells per well were seeded twenty-four hours before transfection. Using the GenJet lipid-based transfection protocol, HEK-293T/17 cells were cotransfected with 400 ng of pSARM4 (wild-type SRV-3) or pSARM-eGFP (SRV-3Δ*env*) or SIVmac239-VIEGFP proviral plasmid along with 50 ng, 100 ng and 200 ng of either HA-tagged human or rhesus tetherin expression constructs to assay for virus restriction. Additionally, 400 ng of plasmids expressing either SIVmac Nef or HIV-1 Vpu or pVSV-G or SRV-3 Env were expressed in *trans* to assess their abilities to rescue viral release. A total of 1 μg of DNA was transfected in each well. The total amount of DNA was normalized by the addition of an empty vector, pcDNA3.1. Forty-eight hours post-transfections, transfected cells were harvested, and virions were pelleted from cultured supernatants. The protocols for the harvest of transfected cells and virus-like-particles (VLPs) pelleting are described in section 2.5.

2.4.2 By stable transfections

All transfections were performed in 6-well plates using the GenJet lipid-based transfection system. For all transfections, the stable 293T cells was seeded at a density of $5x10^5$ cells per well. Each of the 293T-based stable cell lines were either expressing an empty vector (pQCXIP) or the tetherin orthologues from human/cat/dog/squirrel monkey/rhesus macaque/AGM/sooty mangabey/pig-tailed macaque/baboon. To determine any species-specific differences in the pattern of viral restriction, all the stable HEK-293T cells expressing the HA-tagged tetherin orthologues were transfected with 400 ng of SIVmac239-VIEGFP proviral plasmid to assay for virus restriction. Additionally, 400 ng of plasmids expressing either SIVmac Nef or HIV-1 Vpu or SRV-3 Env were expressed in *trans* to assess their abilities to rescue viral release. To map the domain of interaction between tetherin and SRV-3 envelope glycoprotein, the 293T cells stably expressing either HA-tagged human or rhesus tetherin or human-rhesus tetherin reciprocal chimeras or an empty vector (pQCXIP) only were transfected with 400ng of SIVmac239-VIEGFP proviral plasmid to assess viral restriction. 400 ng of either Nef or Vpu or pVSV-G or SRV-3 Env were expressed in *trans* to assay for their abilities to rescue viral release in the presence of the tetherin chimeras. As a control, 293T-based empty vector expressing cell line was transfected with SIVmac239-VIEGFP proviral plasmid to assess viral release in the absence of any tetherin. For all of the above transfections, a total of 1 μg of DNA was transfected in each well. The difference in the total amount of DNA was normalized by the addition of an empty vector, pCGCG expressing an IRES-driven GFP only. Forty-eight hours post-transfections, transfected cells were harvested, and virions were pelleted from cultured supernatants. The protocols

for harvesting transfected cells and virus-like-particles (VLPs) pelleting are described below in section 2.5.

2.5 Cell harvesting technique

2.5.1 Pelleting of virus-like-particles (VLPs)

Two days after transfection, two milliliters of infectious cell culture supernatants containing released virions were collected in 15 milliliters conical tubes and centrifuged at 3000 rpm for 5 minutes at room temperature for decanting the virus-containing supernatants from the cell debris. 1.5 milliliters of the viral supernatants were filtered using 0.45 μm sterile filters. Using a 20% sucrose cushion, the virus-like-particles (VLPs) were pelleted from the filtered viral supernatants by ultracentrifugation at 35,000 rpm for one hour at 4^{o} C. The virions were then lysed by resuspending in 100 μls of 2X Laemmli buffer. The proteins samples were denatured by boiling for five to ten minutes at 99^{o} C and proceeded to SDS-polyacrylamide gel electrophoresis (PAGE).

2.5.2 Preparation of cell lysates

Two days after transfection, the infectious supernatants were removed from the cells, and the transfected cells were washed with 500 μl of PBS and lysed in 300 μl of IP lysis buffer (Thermo Scientific Pierce) at 4^{o} C for thirty minutes. The cell lysates were then cleared from the cell debris by centrifuging at 14,000 rpm for 10 minutes at 4^{o} C. The cleared cell lysates were resuspended in 200 μls of 2X Laemmli buffer. The proteins samples were denatured by boiling at 99^{o} C for five to ten minutes and proceeded to SDS-PAGE.

2.6 Immunoblotting

The viral pellets (obtained from the section 2.5.1) and cell lysates (obtained from the section 2.5.2) were separated on 12% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes by the traditional wet protein transfer method at 100V for an hour. The membranes were blocked in 5% non-fat milk in PBS for two hours at room temperature, and the proteins of interest were probed with the primary antibodies overnight at 4^{o} C. SRV-3/MPMV Gag protein p73 and Capsid protein p27 were probed with anti-MPMV CA rabbit serum (generously provided by Dr. Michaela Rumlova, Institute of Organic chemistry and Biochemistry, Academy of Sciences at the Czech Republic) at a dilution of 1:500 in 5% non-fat milk in PBS. SIVmac239 Gag protein p55 and Capsid protein p27 were detected using mouse monoclonal antibody 55-2F12 (NIH AIDS Reagent Program, Division of AIDS NIAID) at a dilution of 1:1000 in 5% non-fat milk in PBS. Following incubation in the primary antibodies, the membranes were washed four times with PBS containing 0.05% Triton X-100 for fifteen minutes each. The blots were then re-probed with species-specific IgG-HRP-conjugated goat secondary antibodies at a dilution of 1:5000 in 5% non-fat milk in PBS for an hour at room temperature. Thereafter, the blots were again washed six times with PBS containing 0.05% Triton X-100 for fifteen minutes each. The cell lysate blot was stripped for twenty minutes using Restore western blot stripping buffer (Thermo Scientific^{TM} Pierce^{TM}). After two additional five minutes washes in PBS/ 0.05% Triton X-100, the membrane was again blocked in 5% non-fat milk in PBS for an hour. HA-tagged tetherin protein expression was detected using rabbit polyclonal anti-HA-HRP (Thermo Scientific^{TM} Pierce^{TM}) antibody at a dilution of 1:2500 in 5% non-fat milk in PBS and β-actin

expression was probed using mouse monoclonal to beta Actin-HRP conjugated antibody (Abcam) at a dilution of 1:5000 in 5% non-fat milk in PBS. Subsequently, the blots were treated with ECL western blotting detection reagents, and protein bands were visualized using the Biorad ChemiDoc MP imaging system. The viral capsid band intensities in the VLP blots were determined using the Image Lab 4.0.1 software program.

2.7 Downregulation assay

All transfections were performed in 6-well plates using the GenJet lipid-based transfection system. Twenty-four hours prior to transfection, 293T cells stably expressing either HA-tagged human or rhesus tetherin orthologue or an empty vector (pQCXIP) only were seeded at a density of $5x10^5$ cells per well. In order to optimize the amount of DNA required to observe cell surface downregulation of human and rhesus tetherin by Vpu and Nef respectively, titrations were carried out by transfecting differential inputs (500ng, 1 μg, 1.5 μg and 2 μg) of pCGCG-based bicistronic constructs expressing either Vpu or Nef and an IRES-driven GFP. A similar titration was also carried out by transfecting varying amounts (500 ng, 1 μg, 1.5 μg and 2 μg) of pCGCG-SRV-3 *env* plasmid and plasmids expressing pCGCG-based SRV-3 *env* trafficking mutants and cytoplasmic tail alanine scanning mutants to investigate if SRV-3 Env affects cell surface expression of rhesus tetherin. As a control for this experiment, the stable cell lines were also transfected with an empty vector, pCGCG expressing only GFP via an IRES. A total of 2 μg of DNA was used for transfection in each well in this assay. The difference in the total amount of DNA was normalized by the addition of an empty vector, pCGCG expressing an IRES-driven GFP only. Seventy-two hours post-transfection, the cell culture supernatants were

removed and the transfected cells were washed with 500 μl of PBS. The cells were then incubated in 500 μl of Cellstripper solution (Corning) for thirty minutes at room temperature. The action of the Cellstripper was inhibited by the addition of two milliliters of DMEM/10% FBS containing 4 μg/ml of puromycin. The cell suspensions were collected and centrifuged at 1500 rpm for five minutes at room temperature. The supernatants were removed and the cells were washed with five milliliters of PBS. After removing the PBS, the cells were resuspended in 300 μl of PBS, and each of the cell samples were stained with 5 μl of mouse anti-HA-IgG-PE conjugated antibody (Columbia Biosciences) for an hour at room temperature. Following this, the cells were again washed in 300 μl of PBS and fixed in 1% paraformaldehyde PBS. The cells were then analyzed using the BDS FACSAria^{II} SORP flow cytometer, and the results were interpreted using FlowJo 8.7.3 software. At first the cell populations were gated for live cells using the forward and side scatter properties of the cells. In order to exclude the doublets in the analysis, gating was done for single cells using the forward scatter-area (FSC-A) and forward scatter-height (FSC-H) on the live cell population. The single, live cell population was gated for tetherin-GFP^{+} cell populations by using PE on the Y-axis (denotes tetherin) and FITC-A (denotes GFP) on the X-axis. To determine the ability of other simian retroviral envelopes to downregulate the cell surface expression of rhesus tetherin, this same assay was also carried out by transfecting the stable 293T cells expressing HA-tetherin with pCGCG-based SRV-1, 2, 4, 5 *env* and SMRV *env* expression constructs. SRV-3 envelope cytoplasmic tail mutants were also tested in the similar assay to determine if the ability of SRV-3 envelope to downmodulate rhesus tetherin was dependent on the viral envelope trafficking pathway. The remaining cell

harvesting procedure, staining techniques, gating strategy and data analysis method are same as above.

2.8 Co-immunoprecipitation

Twenty-four hours prior to transfection, 293T cells stably expressing either an empty vector (pQCXIP) or HA-tagged human or rhesus tetherin orthologues were seeded at a density of $5x10^5$ cells per well. On the following day, these stable cell-lines were transfected with 2-4 μg of Avi-tagged SRV-3 *env* expression plasmid. Forty-eight hours post-transfection, the cell culture supernatants were removed and the transfected cells were washed with 300 μl of ice-cold PBS. The cells were lysed in 300 μl of IP lysis buffer (Thermo Scientific Pierce) on ice for thirty minutes. The cell lysate was centrifuged at 16,000 x g for eight minutes at 4° C to separate the cell debris. 50 μl of the cell lysate was removed for verification of protein expression by immunoblotting. 25 μl of protein A-Sepharose magnetic beads (New England Biolabs) were added to the remaining 250 μl of the cell lysate and incubated for an hour at 4° C on a rotating platform. The samples were kept on a magnetic rack, and the supernatants were collected into new eppendorf tubes. These samples were incubated with 1 μg of rabbit polyclonal anti-BST2 (ThermoFisher Scientific) antibody for an hour at 4° C on a rotating platform. After an hour, 25 μl of protein A-Sepharose magnetic beads were added to the protein-antibody complex, and the incubation was carried out overnight at 4° C on a rotating platform. The beads were washed thrice with 500 μl IP lysis buffer and resuspended in 35 μls of 2X Laemmli buffer. All samples were boiled for five minutes at 95° C and proceeded to SDS-PAGE and immunoblotting. The immunoblotting procedure described

previously in section 2.6 was repeated here. The expression of SRV-3 Env was detected by using mouse monoclonal anti-Avi tag antibody (Avidity) and tetherin expression was probed with rabbit polyclonal anti-HA-HRP antibody (Thermo Scientific™ Pierce™).

Table 2.1: Primers used for site-directed mutagenesis and PCR amplification of tetherin. The sequence for the HA-epitope is in bold font and the mutations are underlined.

Tetherin/BST2 Primer Name	Primer Sequence
RBST2 HA-tag(134) Fwd	/5' Phos/**TATCCGTATGATGTTCCTGATTATGCT**GCCGGAGGTGGAGGCGACTGAGA-3'
RBST2 HA-tag(134) Rev	/5' Phos/AGACGCGTCCTGAAGCTTGTG-3'
HBST2 HA-tag(131) Fwd	/5' Phos/**TATCCGTATGATGTTCCTGATTATGCT**GCAGAGGTGGAGGCGACTGAGA-3'
HBST2 HA -tag(131) Rev	/5' Phos/AGACGCGTCCTGAAGCTTATG-3'
RBST2 Age1 Fwd	5'-CCTTAACCGGTGCCACCATGGCACCTATTTTGTATGACTATCGC-3'
RBST2 BamH1 Rev	5'-TTGACGGATCCTCACAGCAGCAGAGCGCTCAA-3'
HBST2 Age1 Fwd	5'-CCTTAACCGGTGCCACCATGGCATCTACTTCGTATGACTATTGCAGA-3'
HBST2 BamH1 Rev	5'-TTGACGGATCCCTACTGCAGCAGAGCGCTGAG-3'
Smm BST2 Age1 Fwd	5'-CCTTAACCGGTGCCACCATGGCACCTATTTTGTATGACTATTGC-3'
Smm BST2 BamH1 Rev	5'-TTGACGGATCCTTACAGCAGCAGAGCGCTCAA-3'
AGM-BST2 Age1 Fwd	5'-CCTTAACCGGTGCCACCATGGCACCTATTTTGTATGACTATTGCAAA-3'
AGM-BST2 BamH1 Rev	5'-TTGACGGATCCTCACAGCAGCAGAGCGCC-3'
Baboon BST2 BamH1-1 Rev	5'-TCAAGGGATCCTCACAGCAGCAGA-3'
Dog/Cat BST2 BamH1-1 Rev	5'-TCAAGGGATCCTCAGGCCAG-3'
Dog/Cat/Baboon/Sq. monkey BST2 Age-1	5'-CGTTAACCGGTGCCACCATG-3'
Sq. monkey BST2 BamH1-Rev	5'-TCAAGGGATCCTCAGAGGAGCCGA-3'
HA-rBST2 D15A Fwd	5'-GCCATTTGGAAGGAAGACGGGGAC-3'
HA-rBST2 ΔD15 Fwd	5'-ATTTGGAAGGAAGACGGGGAC-3'
HA-rBST2 D15K/ΔD15 Rev	5'-ATCCATGGGCATTTTGCGATA-3'

Table 2.2: Primers used for site-directed mutagenesis and PCR amplification of viral envelopes. The sequence for the Avi-epitope is in bold font and the mutations are underlined.

Viral *env* Primer name	Primer Sequence
SRV-3 env-pCGCG XbaI Fwd	5'-GAAGCGCGTAGGCCTTCTAGAGCCACCATGAAAGACCCCACCTGTA-3'
SRV-3 env-pCGCG MluI Rev	5'-GTACTCCGGGATCCGACGCGTTTAGCTATGGAAAACTGTCCCG-3'
RD114 env XbaI Fwd	5'-CGTTATCTAGAGCCACCATGAAACTCC-3'
RD114 env MluI Rev	5'-TTGACACGCGTTCAATCCTGAGCTTCTTC-3'
REV env XbaI Fwd	5'-CGTTATCTAGAGCCACCATGGACTGT-3'
REV env MluI Rev	5'-TTGACACGCGTTCATTGACCTAGGGTATCCAT-3'
SMRV env XbaI Fwd	5'-CGTTATCTAGAGCCACCATGCTCT-3'
SMRV env MluI Rev	5'-TTGACACGCGTCTACAGTCTGCCATATTC-3'
BaEV env XbaI Fwd	5'-CGTTATCTAGAGCCACCATGGGAT-3'
BaEV env MluI Rev	5'-TTGACACGCGTTCAATCTTGAGCTTCTTCATCGGT-3'
SRV-1 env XbaI Fwd	5'-CGTTATCTAGAGCCACCATGAACTT-3'
SRV-1 env MluI Rev	5'-TCAAGACGCGTCTATGTTAAGT-3'
SRV-2 env XbaI Fwd	5'-CGTTATCTAGAGCCACCATGACTGTTA-3'
SRV-2 env MluI Rev	5'-TCAAGACGCGTCTACGATACA-3'
SRV-4 env XbaI Fwd	5'-CGTTATCTAGAGCCACCATGAACT-3'
SRV-4 env MluI Rev	5'-TTGACACGCGTTTAAGTTATCTGTAAATATGAGCCACCG-3'
SRV-5 env XbaI Fwd	5'-CGTTATCTAGAGCCACCATGAGC-3'
SRV-5 env MluI Rev	5'-TCAAGACGCGTTTAAATGCT-3'
SRV-3 env CT Y23S Fwd	/5' Phos/5'-AGTCATCGCCTTGAACAAGAAGACAGT-3'
SRV-3 env CT Y23S Rev	/5' Phos/5'-ATGGACTTGTATAGGTTTGGCCTGG-3'
SRV-3 env CT Y35S Fwd	/5' Phos/5'-AGTTTGACCTTAACATAGACGCGTCG-3'
SRV-3 env CT Y35S Rev	/5' Phos/5'-TGAGCCACCACTGTCTTCTTGTTCAA-3'
SRV-3 env Avi-tag Fwd	/5' Phos/5'-**CTCAGAAAATCGAATGGCACGAA**TAGACGCGTCGGATCCCG-3'
SRV-3 env Avi-tag Rev	/5' Phos/5'-**CCTCGAAGATGTCGTTCAGACC**GGCGCCAGCTCCTGTTAAGGTCAAATATGAGCCACC-3 '

CHAPTER 3: RESULTS

3.1 MPMV/SRV-3 envelope glycoprotein is the viral determinant in the host-specific tetherin resistance

The antiviral effector tetherin is targeted by the Vpu protein of HIV-1 and the Nef proteins of simian immunodeficiency viruses (SIVs).[80,81,112] While the focus has been on such complex retroviruses with dedicated tetherin antagonists, it remains unclear as to how simple retroviruses, such as those classified as betaretroviruses and gammaretroviruses, overcome tetherin-mediated restriction. In order to address this gap in knowledge, I first focused on Mason-Pfizer Monkey Virus (MPMV), also known as Simian Retrovirus-3 (SRV-3)

The Type-D simian retroviruses (SRVs) are betaretroviruses that cause pathogenesis and immunodeficiency in Asian macaques. SRV-3, more commonly known as Mason-Pfizer Monkey Virus (MPMV)) is a prototypical type-D betaretrovirus first isolated from the breast carcinoma of female rhesus macaque.[173] Previously, the Bieniasz laboratory reported that SRV-3/MPMV is sensitive to restriction by human tetherin.[92] However, given that the natural host of SRV-3 is the rhesus macaque and together with publishing reports that rhesus macaque tetherin is functional against other retroviruses both in vitro and in vivo,[112] I hypothesized that SRV-3 must be resistant to rhesus macaque tetherin.

In order to test this hypothesis, I first compared the release of SRV-3 virions in the presence of human tetherin and rhesus tetherin. To do this, I transiently cotransfected HEK-293T/17 cells (that do not express any endogenous tetherin), with a full length SRV-3 proviral plasmid and with increasing doses of plasmids expressing either human or rhesus macaque tetherin. Expression of the SRV-3 full-length Gag polyprotein and

capsid protein in the cell lysates and the presence of viral capsid protein in the pelleted supernatant was visualized by western blots (Figure 3.1A). SRV-3 Gag and CA protein expression in the cell lysates remained unaffected in the presence of both human and rhesus tetherin orthologues (Figure 3.1B). However, we observed a decrease in SRV-3 CA protein in the supernatant in the presence of human tetherin but not in the presence of rhesus tetherin. This confirmed that human tetherin restricts release of SRV-3; however, as I hypothesized, SRV-3 was insensitive to restriction by rhesus macaque tetherin (Figure 3.1B). This differential restriction pattern indicated that SRV-3 was specifically adapted to the tetherin homolog of its rhesus macaque host, and may have evolved a mechanism to counteract restriction by rhesus tetherin.

Most complex retroviruses actively evade tetherin-mediated restriction by either encoding an anti-tetherin factor like SIV Nef or HIV-1 Vpu,[80,112] or by deploying their envelope glycoprotein (HIV-2).[93] Since SRV-3 is a simple retrovirus and does not have any accessory genes, I hypothesized that the SRV -3 envelope glycoprotein may antagonize rhesus macaque tetherin. To test this hypothesis, I repeated the single-cycle VLP release using an SRV-3 proviral plasmid containing a deletion of the *env* gene and asked whether the expression of SRV-3 Env was necessary for the release of SRV-3 particles in the presence of rhesus macaque tetherin. The envelope glycoprotein of Vesicular Stomatitis Virus (VSV-G) does not antagonize rhesus tetherin and was used as a negative control.[93] HEK-293T/17 cells were also cotransfected with SRV-3Δ*env* proviral plasmid and SRV-3 Env or VSV-G in the absence of any tetherin orthologues as control to ensure that the viral envelopes were not facilitating a general enhancement in the viral release.

As expected, the co-expression of VSV-G in *trans* failed to rescue the release of SRV-3Δ*env* virus from either the human or rhesus tetherin orthologues (Figure 3.1C). Moreover, the expression of SRV-3 Env in *trans* did not rescue SRV-3Δ*env* virion release in the presence of human tetherin (Figure 3.1C). However, the transient expression of the SRV-3 Env rescued SRV-3Δ*env* virus in the presence of rhesus tetherin (Figure 3.1C), suggesting that it functions as a host-specific tetherin antagonist. These results indicate that SRV-3 is resistant to restriction by rhesus tetherin and overcomes host-specific tetherin-mediated restriction by using its envelope glycoprotein.

Figure 3.1. SRV-3 envelope glycoprotein is the viral determinant in the host-specific tetherin resistance

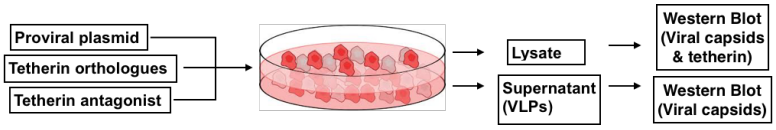
(A) Schematic depiction of the method of single-cycle virus-like-particle (VLP) release assay. This assay was performed by cotransfecting proviral plasmids (SRV-3 or SRV-3Δ*env*) and plasmids expressing tetherin antagonist (such as SRV-3 Env), in HEK-293T cells expressing tetherin orthologues. The SRV-3 Gag and capsid expression in the cell lysates and the presence of viral capsid protein in the pelleted supernatant was visualized by western blots.

(B) Western blot analysis of the transfection of 293T cells with SRV-3 proviral plasmid in the presence or the absence of increasing doses of human or rhesus tetherin.

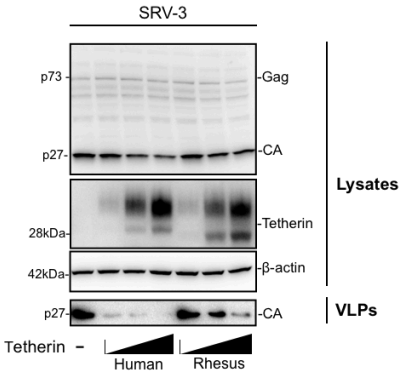
(C) Western blot analysis of the cotransfection of 293T cells with either full-length SRV-3 proviral plasmid or the SRV-3Δ*env* and plasmids expressing SRV-3 Env or VSV-G; transfection was done either in the presence or the absence of increasing doses of human or rhesus tetherin.

In figures (B) and (C)- the SRV-3 Gag and capsid expressions were probed with anti-MPMV capsid rabbit serum and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta-actin was used as a loading control for the lysates.

(A)



(B)



(C)

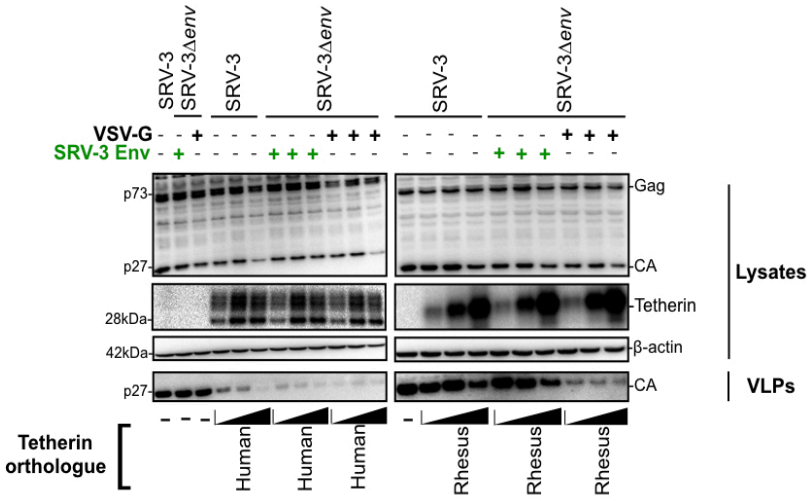


Figure 3.1. SRV-3 envelope glycoprotein is the viral determinant in the host-specific tetherin resistance. Continued.

3.2 SRV-3 envelope rescues a heterologous virus from rhesus tetherin

To determine whether SRV-3 Env is sufficient to antagonize rhesus tetherin, I next asked whether SRV-3 Env is able to rescue an unrelated virus from rhesus tetherin. To do this, I took advantage of another retrovirus found in rhesus macaques, the Simian Immunodeficiency Virus (SIVmac). SIVmac is a primate lentivirus and encodes an accessory protein, Nef, that counteracts restriction by rhesus tetherin.[112] Additionally, some reports suggest SIVmac Env protein may have residual anti-tetherin activity.[158,159]

I therefore asked whether SRV-3 Env expression in *trans* is able to rescue a *nef*- and *env*- deleted variant of SIVmac239 isolate (SIVmac239Δ*nef*Δ*env*) from rhesus tetherin. To test this, I performed the single-cycle VLP release assay by cotransfecting 293T cells with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing the SRV-3 Env, SIVmac239Nef, HIV-1 Vpu, or VSV-G, in the presence and absence of human and rhesus tetherin orthologues. SIV Nef and HIV-1 Vpu antagonize rhesus and human tetherin, respectively, and were used as positive controls.[112] Forty-eight hours post-transfection, transfected cells were harvested and lysed, and virions were pelleted from cultured supernatants by ultracentrifugation. Expression of the SIVmac239 Gag polyprotein and capsid protein in the cell lysate and the presence of the viral capsid expression in the pellets were visualized by western blots.

In the absence of the expression of any of the tetherin antagonists, the release of SIVmac239Δ*nef*Δ*env* virions was inhibited by both human and rhesus tetherin (Figure 3.2). VSV-G did not rescue the virus in the presence of either of the tetherin orthologues (Figure 3.2). Consistent with previous studies, the expression of HIV-1 Vpu in *trans* rescued SIVmac239Δ*nef*Δ*env* virus from human tetherin, but not from rhesus tetherin. In

contrast, SIVmac Nef is known to antagonize rhesus tetherin and not human tetherin; as expected, co-expression of SIVmac Nef rescued virion release in the presence of rhesus tetherin but not from human tetherin. Similar to SIVmac Nef, co-expression of SRV-3 Env rescued the release of SIVmac239Δ*nef*Δ*env* virions from rhesus tetherin, but not from human tetherin. These data strongly suggest that: 1) the SRV-3 envelope is a very potent tetherin antagonist, and that its anti-tetherin function is not specific to the release of SRV-3 only, and 2) the expression of SRV-3 envelope alone is sufficient to antagonize rhesus tetherin and does not require the presence of any other SRV-3 proteins.

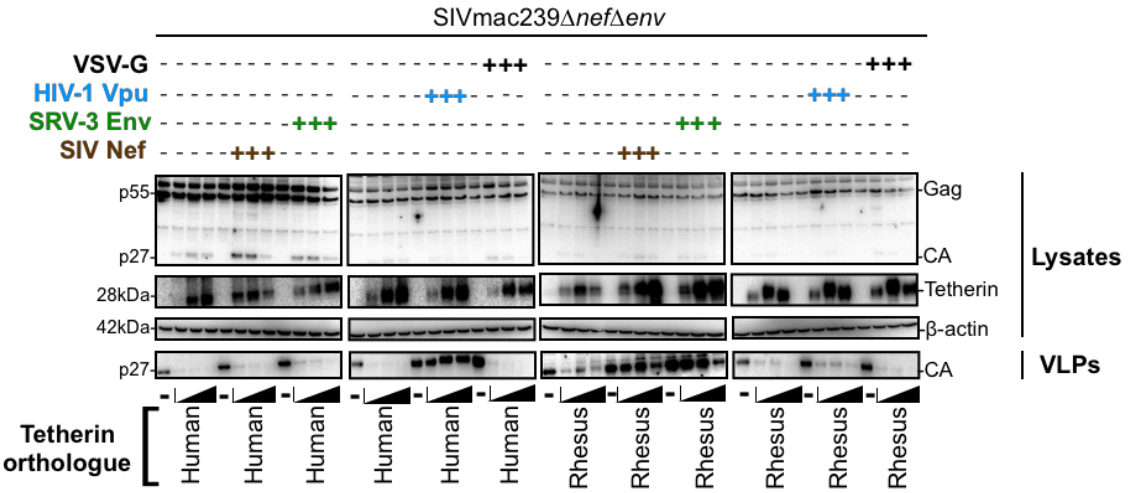
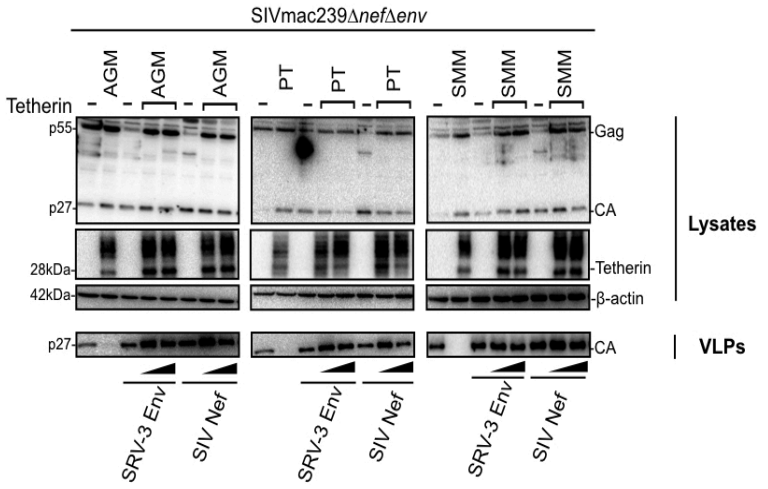


Figure 3.2. SRV-3 envelope rescues a heterologous virus from rhesus tetherin
Western blots showing the results of cotransfection of 293T-based stable cell lines expressing either human tetherin or rhesus tetherin, with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing SIVmac Nef, HIV-1 Vpu, SRV-3 Env, or VSV-G. The SIV Gag and capsid expressions were probed with anti-SIVmac p27 monoclonal antibody and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta-actin was used as a loading control for the lysates.

3.3 SRV-3 Env antagonizes Old world monkey (OWM) tetherin orthologues

We next asked whether SRV-3 Env was able to antagonize restriction by other Old World monkey (OWM) tetherin orthologues. To examine the ability of SRV-3 Env to antagonize other OWM tetherin orthologues, we used HEK-293T cells stably expressing an ectodomain HA-tagged OWM tetherin orthologues from African-green monkey, sooty mangabey and pig-tailed macaques. These cells were cotransfected with SIVmac239Δ*nef*Δ*env* proviral plasmid in the presence of either SRV-3 Env or SIVmac239 Nef or HIV-1 Vpu or VSV-G. In contrast, HIV-1 Vpu did not rescue the virus from any of these OWM tetherins, suggesting that HIV-1 Vpu lacks tetherin-antagonism against the OWM tetherin orthologues. VSV-G also failed to counteract OWM tetherins due to the lack of anti-tetherin function (Figure 3.3B). However, similar to SIVmac239 Nef, SRV-3 Env expression in *trans* rescued SIVmac239Δ*nef*Δ*env* virus from each of the OWM tetherin orthologues that we have tested (Figure 3.3A). Thus, these observations indicate that the SRV-3 envelope glycoprotein appears to function as an antagonist against multiple primate tetherin homologues.

(A)



(B)

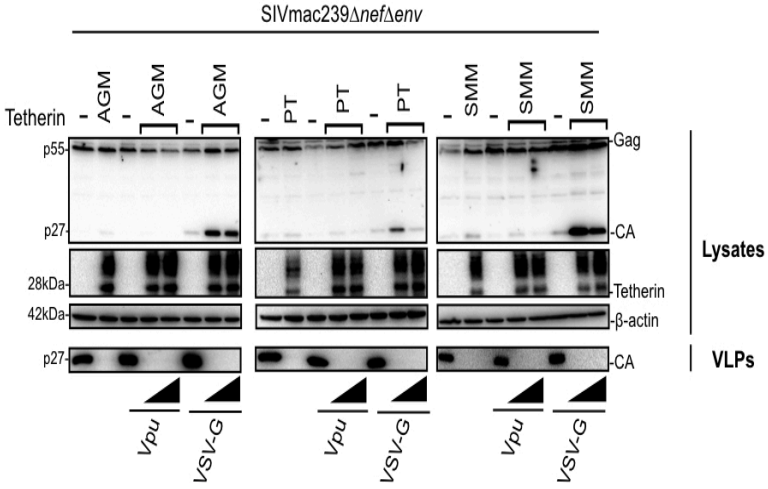


Figure 3.3. SRV-3 Env antagonizes all the OWM tetherin orthologues

(A) Western blot analysis to show the results for the transfection of stable cell lines expressing either pig-tailed macaque (PT) or African-green monkey (AGM) or sooty mangabey (SMM) tetherin orthologues, with SIVmac239Δ*nef*Δ*env* proviral plasmid and SIVmac Nef or SRV-3 envelope expression constructs.

(B) Western blot analysis to show the results for the transfection of the 293T-based stably expressing OWM tetherins with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing HIV-1 Vpu or VSV-G.

In both figures (A) and (B), the SIV Gag and capsid expressions were probed with anti-SIVmac p27 monoclonal antibody and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta-actin was used as a loading control for the lysates.

3.4 The cytoplasmic tail of tetherin influences sensitivity to SRV-3 envelope

To map the determinants of differential tetherin-antagonism by the SRV-3 envelope, I used a panel of 293T-based cell lines that were engineered to stably express HA-tagged human and rhesus tetherin reciprocal chimeras. The reciprocal tetherin chimeras were constructed by either swapping the cytoplasmic tail or the transmembrane domain of rhesus tetherin in human tetherin and vice versa.[112] These stable cell lines were cotransfected with SIVmac239Δ*nef*Δ*env* proviral plasmid and differential inputs of either SRV-3 *env*, or SIVmac239 *nef*, or HIV-1 *vpu*, or VSV-g expression constructs. In the absence of any tetherin antagonist, the release of SIVmac239Δ*nef*Δ*env* virus was strongly inhibited by both wild-type rhesus tetherin and wild-type human tetherin and by all of the human-rhesus chimeric tetherin proteins (Figures 3.4A and 3.4B). As positive controls, we used SIVmac Nef and HIV-1 Vpu. Previous studies have shown that Vpu antagonizes human tetherin by interacting with the transmembrane domain of human tetherin while SIVmac239 Nef counteracts rhesus tetherin by targeting the cytoplasmic tail of rhesus tetherin [112]. Consistent with these findings, we also observed that Vpu was able to rescue SIVmac239Δ*nef*Δ*env* virus in the presence of those tetherin chimeras, that encode the human tetherin transmembrane domain (Figure 3.4A). We further observed that SIVmac239 Nef was able to promote virion release in the presence of only those tetherin chimeras that contained the rhesus tetherin cytoplasmic tail (Figure 3.4A).

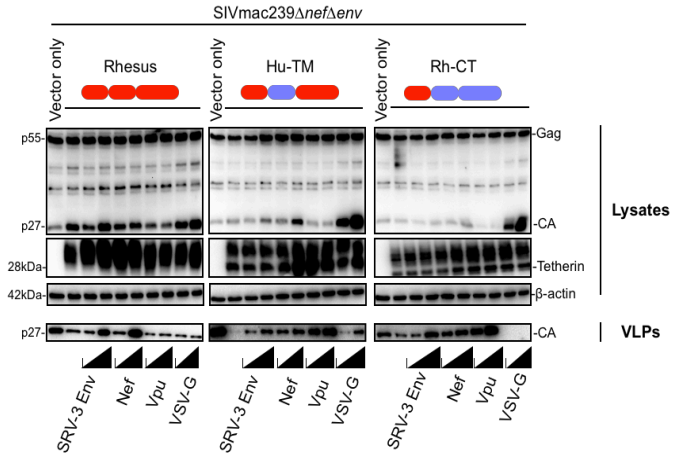
SRV-3 Env was unable to rescue virions in the presence of the Hu-CT and Rh-TM tetherin chimeras, both of which encode the cytoplasmic tail of human tetherin (Figure 3.4B). In contrast, SRV-3 Env rescued SIVmac239Δ*nef*Δ*env* virions in the presence of both the Hu-TM and Rh-CT tetherin chimeras, both of which encode the

cytoplasmic tail of rhesus tetherin. These findings indicate that the cytoplasmic tail of rhesus tetherin is the key determinant of SRV-3 envelope-mediated tetherin-antagonism.

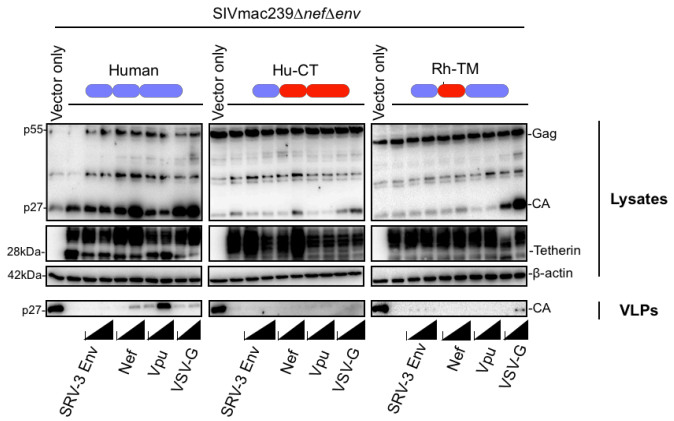
I next sought to identify specific residues in the rhesus tetherin cytoplasmic tail that account for the differential restriction pattern. Comparative sequence analysis of human and rhesus tetherin cytoplasmic tails revealed a difference of eleven amino acids, including a segment of five consecutive residues, $_{14}$GDIWK$_{18}$, found in the rhesus tetherin cytoplasmic tail but absent from the human tetherin (Figure 3.4D). Previous study by Jia et al., has shown that the SIVmac Nef targets these five residues on rhesus tetherin cytoplasmic tail.[112] Given the similar patterns of antagonism displayed by the SIVmac Nef and SRV-3 envelope, we hypothesize that these same residues may affect recognition by SRV-3 envelope. To test this, we performed the single-cycle VLP release assay using cell lines engineered to stably express human and rhesus tetherin reciprocal mutants (Hu-GDIWK and RhΔGDIWK) (Figure 3.4D). Similar to SIVmac Nef, SRV-3 Env was unable to rescue virion release in the presence of the rhesus tetherin mutant lacking these five residues (RhΔGDIWK). In contrast, SRV-3 Env rescued virion release from the human tetherin mutant (Hu-GDIWK) in which these five residues have been restored (Figure 3.4E). These findings reveal that the binding sites for SIVmac Nef and SRV-3 Env overlap and involve some or all of the residues from position 14 to 18 in the cytoplasmic tail of rhesus tetherin.

Figure 3.4: The cytoplasmic tail of tetherin influences sensitivity to SRV-3 envelope
(A) HA-tagged human (highlighted in blue)-rhesus (highlighted in red) tetherin reciprocal chimeras were constructed by swapping the transmembrane domain and cytoplasmic tail of rhesus tetherin in human tetherin and vice versa. 293T based stable cell-lines were generated for stably expressing these tetherin chimeras. Western blots showing the results of cotransfection of the stable cell lines {expressing either rhesus tetherin or rhesus tetherin with human tetherin transmembrane domain (Hu-TM) or human tetherin with rhesus tetherin cytoplasmic tail (Rh-CT)} with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing SIVmac Nef, HIV-1 Vpu, SRV-3 Env, or VSV-G.
(B) Western blots showing the results of cotransfection of stable cell lines {expressing either human tetherin or human tetherin with rhesus tetherin transmembrane domain (Rh-TM) or rhesus tetherin with human tetherin cytoplasmic tail (Hu-CT)} with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing SIVmac Nef, HIV-1 Vpu, SRV-3 Env, or VSV-G.
(C) Western blots showing the results of cotransfection of an empty vector expressing cell lines, with SIVmac239Δ*nef*Δ*env* proviral plasmid and either SIVmac Nef, or HIV-1 Vpu, or SRV-3 Env, or VSV-G.
(D) Sequence alignment showing the amino acid differences in the cytoplasmic tail of human and rhesus tetherin. Dashes indicate deletions; amino acid differences are highlighted in red. Rhesus and human tetherin mutants were created by introducing $_{14}$GDIWK$_{18}$ motif in human tetherin CT (Hu+GDIWK) and deleting the same from the rhesus tetherin CT (RhΔGDIWK).
(E) Western blot analysis showing the results of cotransfection of stable cell lines expressing the rhesus and human tetherin reciprocal mutants, with SIVmac239Δ*nef*Δ*env* proviral plasmid and increasing inputs of either SRV-3 Env or SIVmac Nef expression constructs. The differences in the restriction patterns of the tetherin mutants in the presence of SRV-3 envelope are highlighted by the red boxes.
In figures (A), (B), (C) and (E), the SIV Gag and capsid expressions were probed with anti-SIVmac p27 monoclonal antibody and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta actin was used as a loading control for the cell lysates.

(A)



(B)



(C)

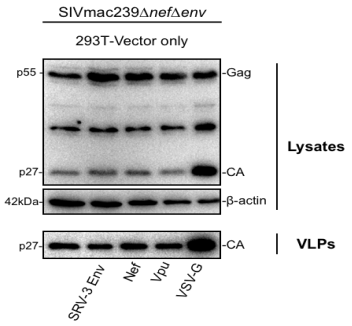
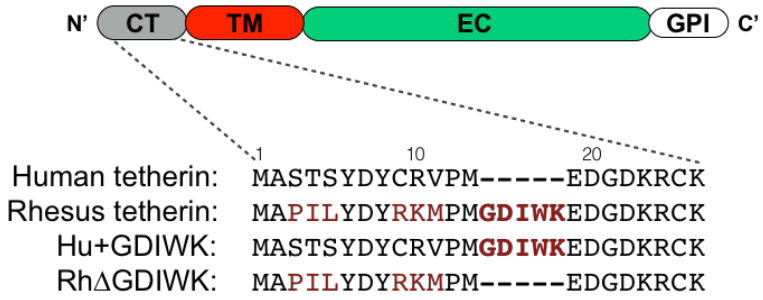


Figure 3.4. The cytoplasmic tail of tetherin influences sensitivity to SRV-3 envelope. Continued.

(D)



(E)

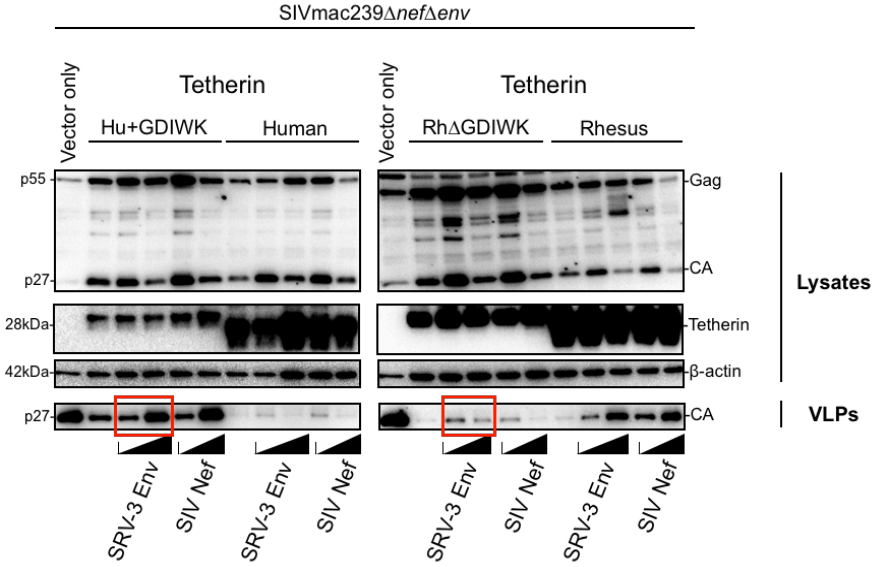


Figure 3.4. The cytoplasmic tail of tetherin influences sensitivity to SRV-3 envelope. Continued.

3.5 SRV-3 envelope glycoprotein physically associates with rhesus tetherin

To determine whether antagonism by SRV-3 envelope involves a direct physical interaction with tetherin, we performed a co-immunoprecipitation assay. Tetherin was immunoprecipitated from the cell lysates using a polyclonal anti-BST2 antibody. We observed that SRV-3 Env was efficiently pulled down upon immunoprecipitation of the wild-type rhesus tetherin (Figure 3.5). Additionally, SRV-3 Env was also co-immunoprecipitated with the human tetherin mutant (Hu-GDIWK) that had the $_{14}$GDIWK$_{18}$ residues of rhesus tetherin in its cytoplasmic tail. However, as expected there was no detectable physical association between SRV-3 Env and the wild-type human tetherin or the rhesus tetherin mutant, RhΔGDIWK (Figure 3.5). Therefore, this data confirms that the SRV-3 Env physically interacts with rhesus tetherin, and that the interaction likely involves the $_{14}$GDIWK$_{18}$ motif in the cytoplasmic tail of rhesus tetherin.

3.6 The envelope glycoproteins of simian retroviruses (SRVs) are tetherin antagonists

SRV-3 is a prototypical type-D retrovirus and this group consists of several other simian retroviruses (SRV-1, -2, -4 and -5) having similar genomes and overlapping host ranges.[13] Hence, we wanted to ask if the observed anti-tetherin function of the SRV-3 envelope was conserved among the envelope glycoproteins of these related viruses. To address this question, I tested the envelope glycoproteins of SRV-1, -2, -4 and -5 in the single cycle-VLP release assay for the ability to rescue SIVmac239Δ*nef*Δ*env* virion release in the presence of the human and rhesus tetherin orthologues. Like SIVmac Nef, the SRV envelope glycoproteins that I have tested, exhibited similar pattern of tetherin-

antagonism against rhesus tetherin. The transient expression of the envelope glycoproteins from SRV-1 through SRV-5 rescued SIVmac239Δ*nef*Δ*env* virion release in the presence of rhesus tetherin but not in the presence of human tetherin (Figure 3.6A). Thus, these reveal that tetherin-antagonism is a conserved trait of the envelope glycoproteins of the SRVs of Asian macaques.

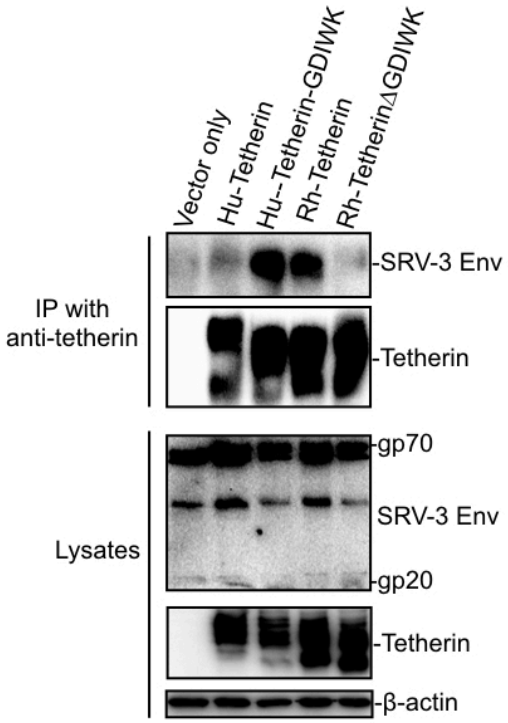


Figure 3.5. SRV-3 envelope glycoprotein physically associates with rhesus tetherin
Co-immunoprecipitation assay of stable cell lines expressing either an empty vector or human and rhesus tetherin orthologues or human and rhesus tetherin reciprocal mutants that were transfected with plasmid expressing a C-terminal Avi-tagged SRV-3 *env*. Tetherin was immunoprecipitated with an anti-BST2 polyclonal antibody. The immunoprecipitation was analyzed by western blotting. The expression of SRV-3 envelope and tetherin in the whole cell lysates were visualized by western blotting. SRV-3 envelope expression was probed with anti-Avi tag antibody and tetherin expression was probed with anti-HA antibody.

Figure 3.6. The envelope glycoproteins of simian retroviruses (SRVs) are tetherin antagonists

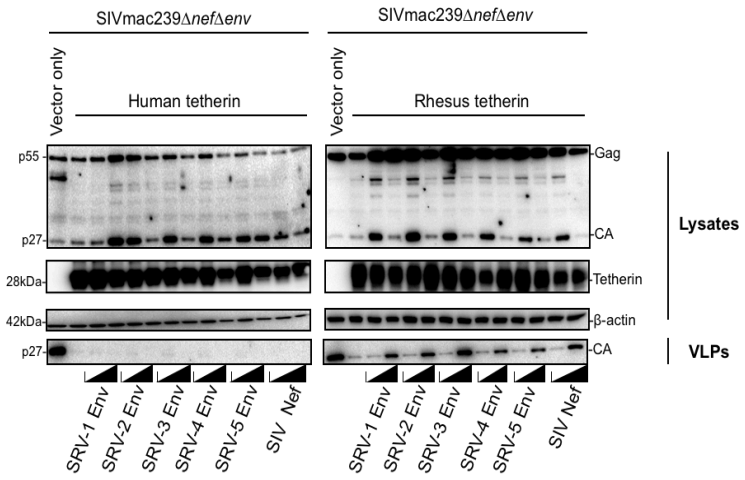
(A) Western blot analysis showing the results of cotransfection of 293T-based stable cell lines expressing rhesus or human tetherin orthologues, with SIVmac239Δ*nef*Δ*env* proviral plasmid and differential doses of either SRV-3 Env or SIVmac Nef.

(B) Western blot analysis showing the results of cotransfection of the human and rhesus tetherin expressing stable cell lines with SIVmac239Δ*nef*Δ*env* proviral plasmid and increasing inputs of either HIV-1 Vpu or VSV-G.

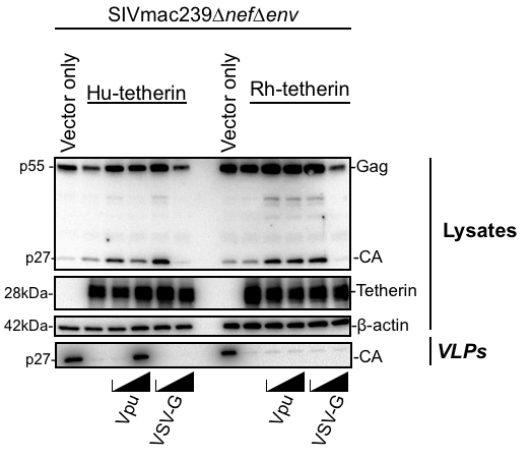
(C) Western blots showing the results of cotransfection of an empty control cell lines, with SIVmac239Δ*nef*Δ*env* proviral plasmid and either SIVmac *nef*, or HIV-1 *vpu*, or VSV-*g*, or SRV-1 through SRV-5 *env* expression constructs.

In figures (A), (B) and (C), the SIV Gag and capsid protein expressions were probed with anti-SIVmac p27 monoclonal antibody and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta actin was used as a loading control for the cell lysates.

(A)



(B)



(C)

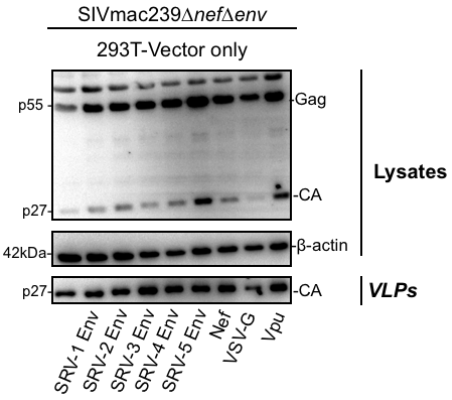


Figure 3.6: The envelope glycoproteins of simian retroviruses (SRVs) are tetherin antagonists. Continued.

3.7 Simian retroviral envelopes downmodulate rhesus tetherin from the cell surface

We next wanted to investigate the tetherin- evasion mechanism employed by the SRV envelopes to counteract restriction by rhesus tetherin. Previously, the tetherin antagonists of complex retroviruses like SIVmac Nef, HIV-1 Vpu and HIV-2 Env were shown to downregulate their respective host tetherins from the cell surface.[93,112,133] To determine if SRV Envs affected the cell-surface levels of rhesus tetherin, we used a two-color flow cytometry assay.[112] At first, a series of titrations were performed by using cell lines that were engineered to stably express either the human or the rhesus tetherin having an ectodomain HA-epitope. These stable cell lines were transfected with differential inputs of vector expressing tetherin antagonist (either SIVmac Nef or HIV-1 Vpu or SRV-3 Env) and an IRES-driven GFP. Since SIVmac Nef and HIV-1 Vpu were used as controls against rhesus and human tetherin respectively, we wanted to optimize the amount of DNA required to observe cell surface downregulation of human and rhesus tetherin by HIV-1 Vpu and SIVmac Nef respectively. The transfected cells were stained for cell-surface tetherin expression and analyzed by flow cytometry. At first, the cell populations were gated for live cells using the forward and side scatter properties of the cells. In order to exclude the doublets in the analysis, gating was done for single cells using the forward scatter-area (FSC-A) and forward scatter-height (FSC-H) on the live cell population. The single, live cell population was gated for tetherin-GFP$^+$ cell populations indicated by PE on the Y-axis and FIT-C on the X-axis (Figure 3.7A). The degree of cell surface downregulation of tetherin was determined by the median fluorescence intensity (MFI) values (Table 3.1). We noticed that an optimal amount of 500ng of Nef and 1 μg of Vpu was necessary to observe a 40% reduction in the cell

surface expression of rhesus and human tetherin respectively (Figure 3.7B and Table 3.1). In contrast, we observed that increasing inputs of SRV-3 Env resulted in an increase in the magnitude of cell surface downregulation of rhesus tetherin (Figure 3.7C and Table 3.1). As expected, the expression of SRV-3 envelope did not reduce cell surface levels of human tetherin.

We conducted the same assay by transfecting the cells expressing either an ectodomain HA-tagged human tetherin or rhesus tetherin, with bicistronic constructs expressing the envelope glycoproteins of SRV-1, -2, -3, -4 and -5 and an IRES-driven GFP. We also tested the envelope glycoprotein of SMRV (a new world monkey SRV) for its ability to downmodulate tetherin from the cell surface. Although the SRV envelopes did not affect the cell surface expression of human tetherin, they did result in a 30%-60% reduction in the cell surface levels of rhesus tetherin (Figure 3.7D and Table 3.2). Therefore, our data suggest that in the absence of a dedicated tetherin antagonist, the envelope glycoproteins of simian retroviruses have evolved to evade tetherin-mediated restriction by reducing the cell surface rhesus tetherin levels.

3.8 The ability of SRV-3 envelope to downmodulate cell surface expression of rhesus tetherin is independent of its trafficking pathway

In 2009, Tortorec et al., showed that the endocytosis motif GYxxθ in the cytoplasmic tail of HIV-2 Env was necessary for reducing cell surface levels of human tetherin and sequestering it within the TGN.[93] SRV-3 Env has a dileucine and a tyrosine-motifs in its cytoplasmic tail, which were shown to play critical roles in envelope trafficking.[33] The leucine at position 3 in the SRV-3 Env cytoplasmic tail was reported to

be necessary for the anterograde transport and a tyrosine at position 23 in the cytoplasmic tail of SRV-3 Env was critical for the recycling of the SRV-3 envelope glycoprotein.[33] Mutations of both leucine and tyrosine to serine alter the trafficking pathway of the SRV-3 envelope glycoproteins. The cytoplasmic tail of SRV-3 envelope also harbors a second tyrosine at position 35 that was shown to be indispensable for Env trafficking.[33] Hence, I wanted to investigate whether the mutations in the dileucine and the tyrosine-motifs in the SRV-3 Env cytoplasmic tail abrogated its ability to downmodulate rhesus tetherin from the cell surface. I performed the two-color flow cytometry assay by transfecting stable cell lines engineered to express either an ectodomain-HA-tagged human or rhesus tetherin orthologue, with bicistronic plasmids expressing either the wild-type SRV-3 envelope or the SRV-3 envelope cytoplasmic tail trafficking mutants (L3S or Y23S or Y35S or L3S/Y23S) and an IRES-driven GFP. The stable cell lines were also cotransfected with bicistronic constructs expressing either SIVmac Nef or HIV-1 Vpu and an IRES-driven GFP to serve as reciprocal controls against rhesus and human tetherin respectively. The transfected cells were stained for cell-surface tetherin expression and the degree of cell surface downregulation of tetherin was determined by the median fluorescence intensity (MFI) values (Table 3.3). Interestingly, mutating the SRV-3 envelope trafficking signals in its cytoplasmic tail did not abrogate the ability of SRV-3 envelope to downregulate rhesus tetherin from the cell surface (Figure 3.8B). The SRV-3 envelope cytoplasmic tail mutants L3S and Y23S mutants resulted in a 30% reduction of cell surface rhesus tetherin levels while the Y35S and L3S/Y23S mutants significantly downregulated cell surface rhesus tetherin levels to approximately 50% (Table 3.3). However, similar to the wild-type SRV-3 envelope, the SRV-3 envelope

trafficking mutants had no impact on the cell surface expression of human tetherin (Figure 3.8B and Table 3.3). The sequences of all the SRV-3 Env CT trafficking mutants used in this study are listed in Figure 3.8A.

Since mutations in the SRV-3 envelope trafficking signals did not affect its ability to downregulate cell surface rhesus tetherin levels, I conducted alanine scanning mutagenesis to identify the residues in the SRV-3 Env cytoplasmic tail that might be critical for downregulating the cell surface expression of rhesus tetherin. I did a comparative sequence analysis of the cytoplasmic tails of the different simian retroviral envelope glycoproteins and found some conserved motifs within them. These include the dileucine motif (L_3M_4), a tyrosine-based motif at position 23 ($Y_{23}HRL_{26}$), the conserved acidic patch ($E_{27}QED_{30}$) and a second tyrosine-based motif at position 35 ($Y_{35}LTLT_{39}$). These individual motifs in the cytoplasmic tail of SRV-3 envelope were replaced with stretches of alanines: L_3M_4 were mutated to A_3A_4, $Y_{23}HRL_{26}$ were mutated to A_{23}-A_{26}, $Y_{35}LTLT_{39}$ were mutated to A_{35}-A_{39} and $E_{27}QED_{30}$ were mutated to A_{27}-A_{30}. The sequences of these SRV-3 Env CT alanine scanning mutants are listed in Figure 3.8C. The two-color flow cytometry assay was carried out by transfecting the HA-tagged human and rhesus tetherin expressing stable cell lines with plasmids expressing either the wild-type SRV-3 envelope or the SRV-3 envelope cytoplasmic tail alanine scanning mutants. The stable cell lines were also cotransfected with plasmids expressing either SIVmac Nef or HIV-1 Vpu to serve as reciprocal controls against rhesus and human tetherin respectively. The transfected cells were stained for cell-surface tetherin expression and the degree of cell surface downregulation of tetherin was determined by the median fluorescence intensity (MFI) values (Table 3.4).

Similar to the wild-type SRV-3 envelope, most of the SRV-3 envelope cytoplasmic tail alanine scanning mutants reduced cell surface rhesus tetherin levels by approximately 40% (Figure 3.8D and Table 3.4). In contrast, the SRV-3 Env CT alanine scanning mutants had little or no impact on the cell surface levels of human tetherin (Figures 3.8D and Tables 3.4). Overall the data from this assay indicate that the ability of SRV-3 envelope to downregulate cell surface expression of rhesus tetherin is not dependent on the known trafficking mutants in the cytoplasmic tail of the envelope. This observation also raises the possibility that the interaction between rhesus tetherin and SRV-3 envelope might take place within the secretory pathway before both the proteins reach the cell surface. It is possible that instead of depleting rhesus tetherin from the cell surface, the SRV-3 Env might be actually sequestering newly-synthesized tetherin in the trans-Golgi network (TGN) and preventing it from reaching the cell surface. Further work using fluorescence imaging technique could help to localize the SRV-3 envelope-tetherin interaction.

Figure 3.7. Simian retroviral envelopes downmodulate rhesus tetherin from the cell surface

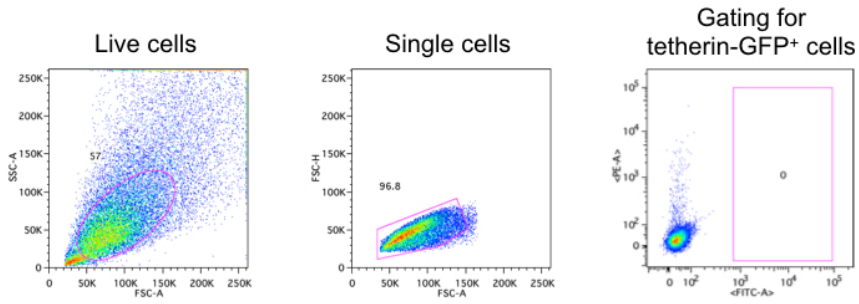
(A) Representative dot plots showing the gating strategy used to analyze flow cytometry data. The cell populations were gated for live cells using the forward and side scatter properties of the cells. In order to exclude the doublets in the analysis, gating was done for single cells using the forward scatter-area (FSC-A) and forward scatter-height (FSC-H) on the live cell population. The single, live cell population was gated for tetherin-GFP$^+$ cell populations indicated by PE on the Y-axis and FIT-C on the X-axis.

(B) A two-color flow cytometry assay was performed to optimize the amount of Nef and Vpu plasmids required to observe downregulation of cell surface levels of rhesus and human tetherin. Stable 293T cells expressing an ectodomain HA-tag rhesus or human tetherin were transfected with differential inputs (500 ng, 1 μg, 1.5 μg and 2 μg) of vector expressing tetherin antagonist (either SIVmac Nef or HIV-1 Vpu), that has an IRES-driven GFP. Cells were also transfected with an empty vector (lacking any tetherin antagonist) as a control. Seventy-two hours after transfection, the cells were stained for cell surface tetherin expression with an anti-HA-IgG-PE conjugated antibody and analyzed using flow cytometry. X-axis represents tetherin and Y-axis denotes tetherin-antagonist-GFP (SIVmac Nef or HIV-1 Vpu). The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells are shown in table 3.1.

(C) Flow cytometry analysis of the transfections of stable cell lines expressing either HA-tagged rhesus or human tetherin orthologues, with increasing inputs (500 ng, 1 μg, 1.5 μg and 2 μg) of bicistronic plasmids expressing SRV-3 envelope and an IRES-driven GFP. X-axis represents tetherin and Y-axis denotes tetherin-antagonist-GFP (SRV-3). The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells are shown in table 3.1.

(D) Flow cytometry analysis of the transfections of stable cells expressing either HA-tagged rhesus or human tetherin orthologues, with 2 μg of bicistronic plasmids expressing simian retroviral envelope glycoproteins and an IRES-driven GFP. Two days after transfection, the cells were stained for cell surface tetherin expression with an anti-HA-IgG-PE conjugated antibody and analyzed using flow cytometry. X-axis represents tetherin and Y-axis denotes tetherin-antagonist-GFP. The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells are shown in table 3.2.

(A)



(B)

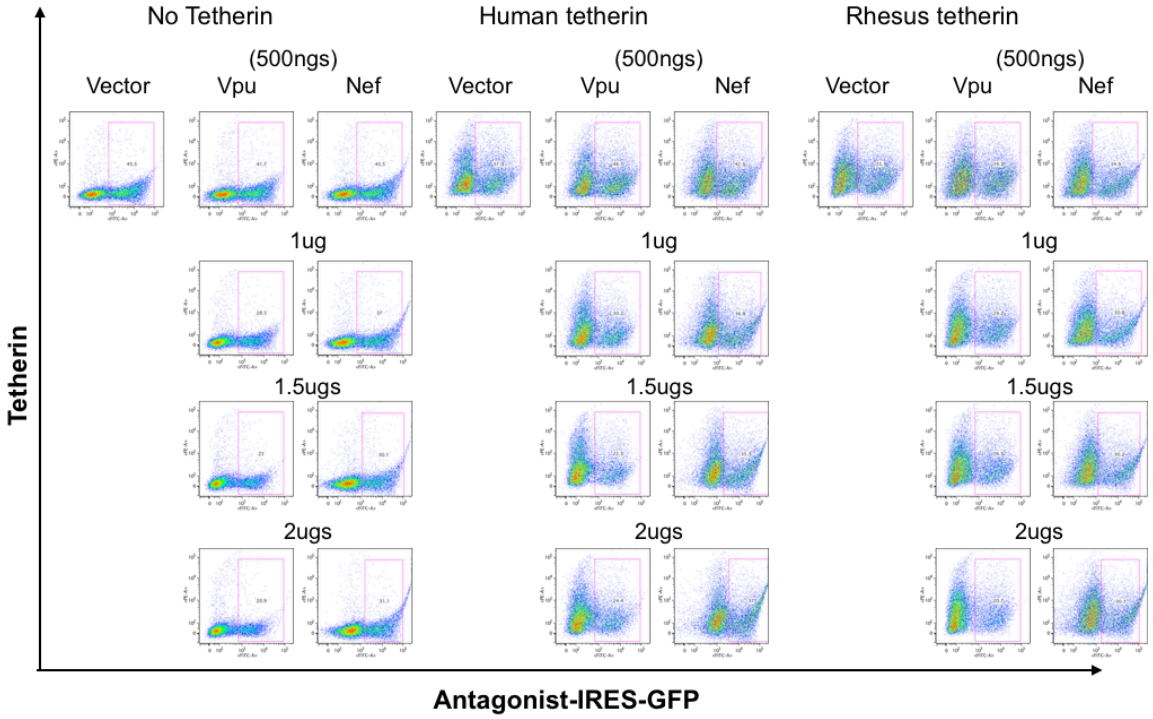
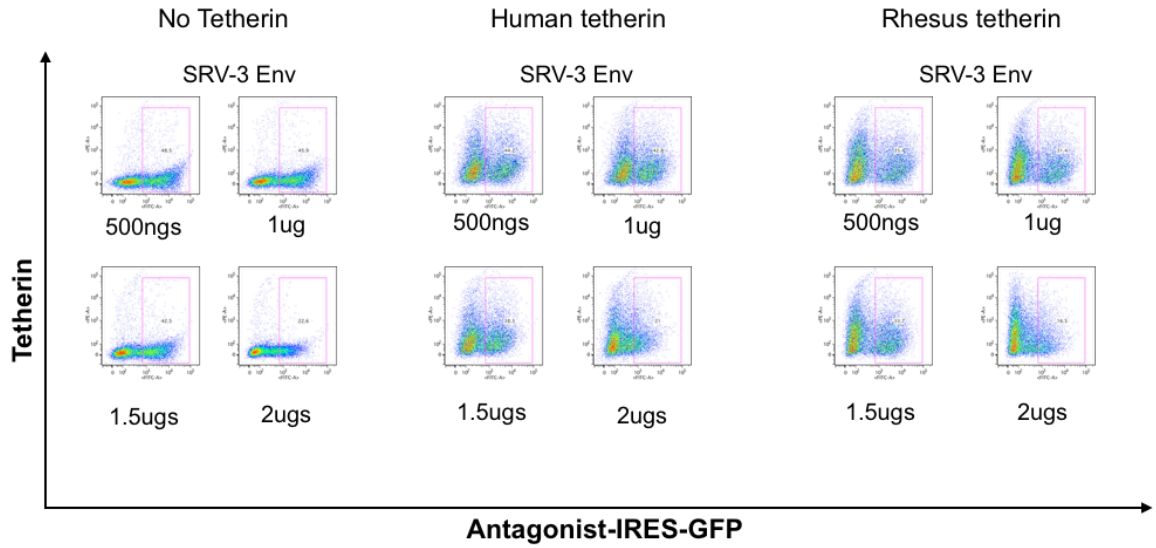


Figure 3.7. Simian retroviral envelopes downmodulate rhesus tetherin from the cell surface. Continued.

(C)



(D)

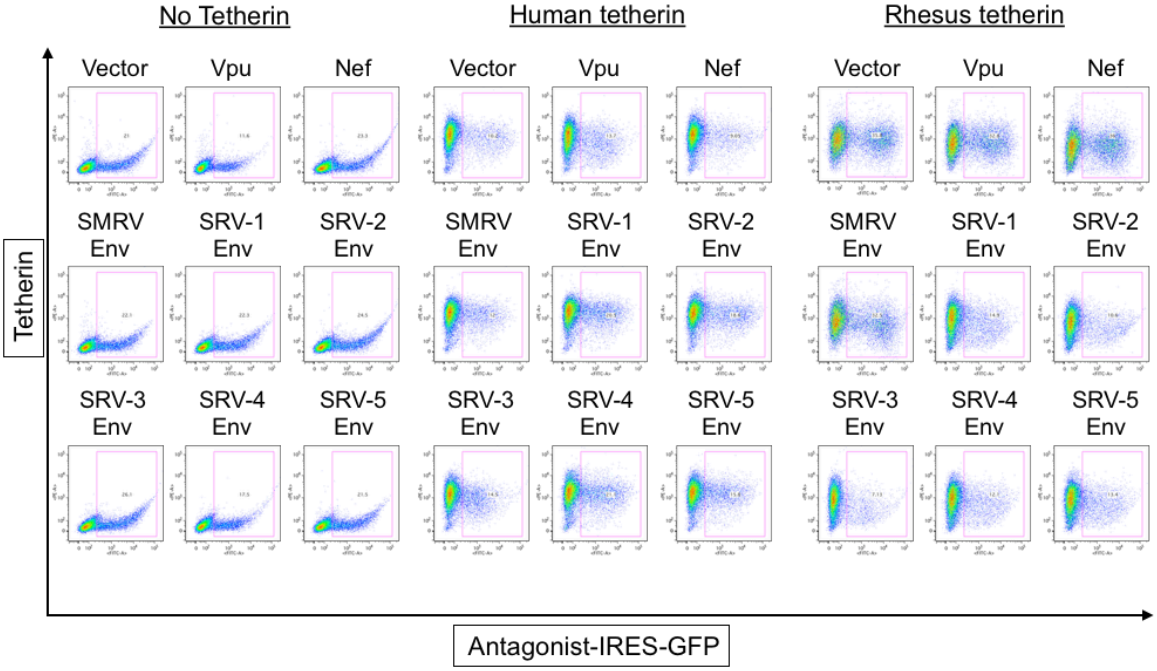


Figure 3.7. Simian retroviral envelopes downmodulate rhesus tetherin from the cell surface. Continued.

Table 3.1. The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells for analyzing the downregulation of cell surface tetherin expressions by HIV-1 Vpu, SIVmac Nef and SRV-3 envelope (Env) glycoprotein

Tetherin Antagonist	Human tetherin		Rhesus tetherin	
	MFI	% change in MFI	MFI	% change in MFI
Vector	187	100.0	249	100.0
Vpu				
500ngs	144	77.0	216	86.7
1.0µg	118	63.1	207	83.1
1.5µgs	167	89.3	218	87.6
2.0µgs	155	82.9	228	91.6
Nef				
500ngs	146	78.1	148	59.4
1.0µg	135	72.2	166	66.7
1.5µgs	167	89.3	194	77.9
2.0µgs	187	100.0	203	81.5
SRV-3 Env				
500ngs	188	100.5	190	76.3
1.0µg	158	84.5	160	64.3
1.5µgs	154	82.4	137	55.0
2.0µgs	156	83.4	121	48.6

Table 3.2. The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells for analyzing the downregulation of cell surface tetherin expressions by the simian retroviral envelope glycoprotein

Tetherin Antagonist	Human tetherin		Rhesus tetherin	
	MFI	% change in MFI	MFI	% change in MFI
Vector	1121	100.0	893	100.0
Vpu	684	61.0	709	79.4
Nef	1236	110.3	506	56.7
SMRV Env	1379	123.0	595	66.6
SRV-1 Env	1686	150.4	579	64.8
SRV-2 Env	1466	130.8	435	48.7
SRV-3 Env	996	88.8	336	37.6
SRV-4 Env	1422	126.9	583	65.3
SRV-5 Env	1472	131.3	540	60.5

Figure 3.8. The ability of SRV-3 envelope to downmodulate cell surface expression of rhesus tetherin is independent of its trafficking pathway

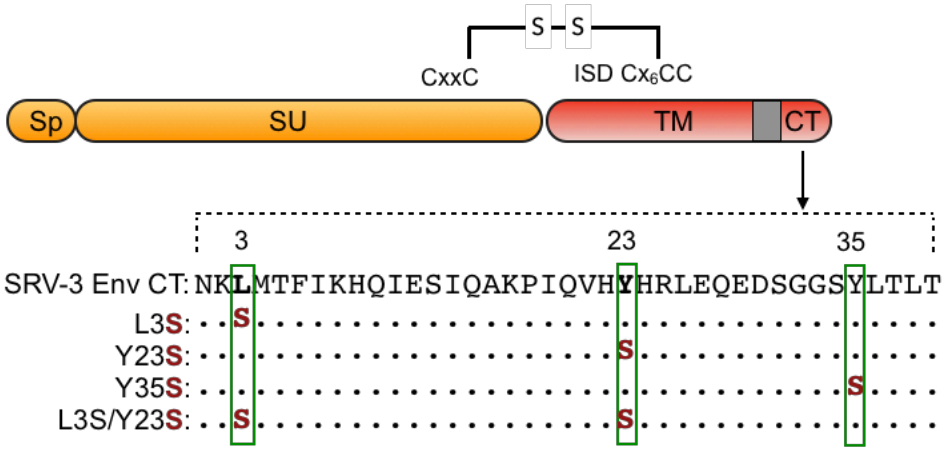
(A) Schematic showing the sequences of the SRV-3 envelope trafficking mutants. The amino acid changes are highlighted in red and the amino acid position is highlighted with a green box.

(B) A two-color flow cytometry assay was conducted with SRV-3 envelope trafficking mutants to investigate if the mutations in the trafficking motifs impede the ability of the SRV-3 envelope to downregulate the cell surface levels of rhesus tetherin. Stable cells expressing HA-tagged rhesus or human tetherin orthologues were transfected with 2 μg of bicistronic plasmids expressing SRV-3 trafficking mutants and an IRES-driven GFP. SIVmac Nef and HIV-1 Vpu were used as controls against rhesus and human tetherin respectively. Two days after transfection, the cells were stained for cell surface tetherin expression with an anti-HA-IgG-PE conjugated antibody and analyzed using flow cytometry. X-axis represents tetherin and Y-axis represents tetherin-antagonist-GFP. The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells are shown in table 3.3.

(C) Schematic showing the sequences of the SRV-3 envelope cytoplasmic tail mutants used in the alanine scanning mutagenesis study. The amino acid changes are highlighted in red.

(D) Flow cytometry analysis of transfections of cells stably expressing HA-tagged rhesus or human tetherin orthologues, with the SRV-3 envelope cytoplasmic tail alanine scanning mutants. The cells were stained with an anti-HA-IgG-PE conjugated antibody for cell surface tetherin expression. The X-axis represents tetherin and Y-axis represents tetherin-antagonist-GFP. The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells are shown in table 3.4.

(A)



(B)

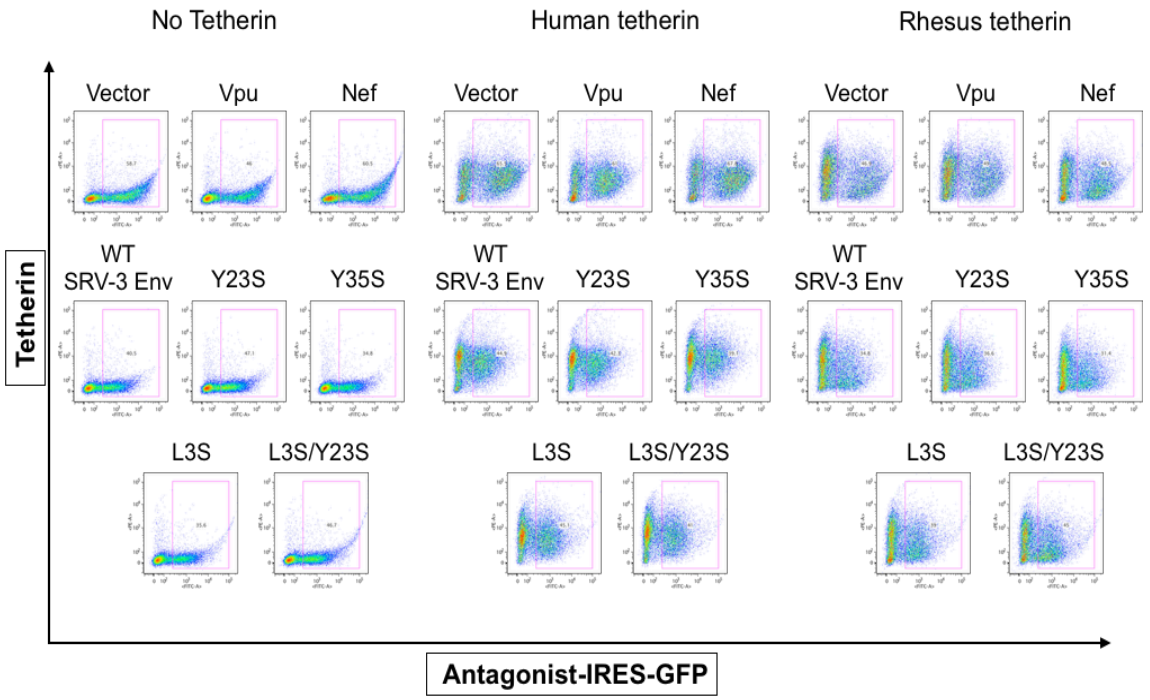
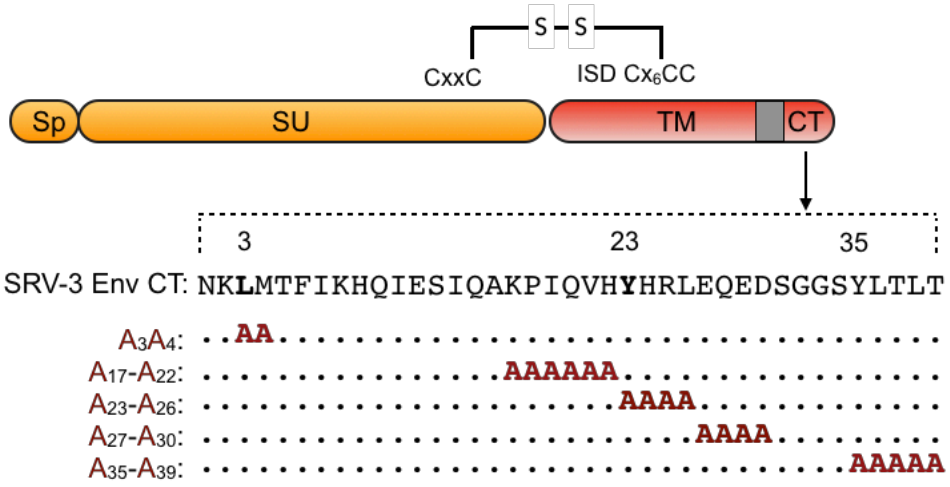


Figure 3.8. The ability of SRV-3 envelope to downmodulate cell surface expression of rhesus tetherin is independent of its trafficking pathway. Continued.

(C)



(D)

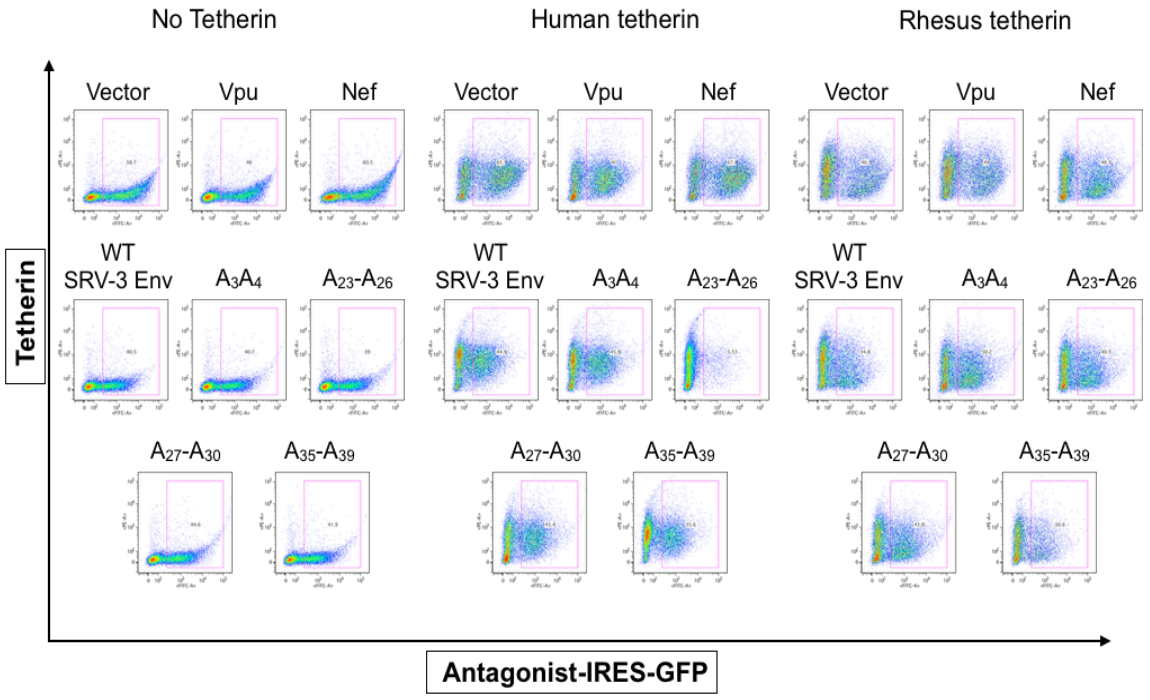


Figure 3.8. The ability of SRV-3 envelope to downmodulate cell surface expression of rhesus tetherin is independent of its trafficking pathway. Continued.

Table 3.3. The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells for analyzing the downregulation of cell surface tetherin expressions by the SRV-3 envelope trafficking mutants

Tetherin Antagonist	Human tetherin		Rhesus tetherin	
	MFI	% change in MFI	MFI	% change in MFI
Vector	360	100.0	291	100.0
Vpu	268	74.4	394	135.4
Nef	318	88.3	192	66.0
WT SRV-3 Env	543	150.8	196	67.4
Y23S	492	136.7	212	72.9
Y35S	381	105.8	165	56.7
L3S	366	101.7	207	71.1
L3S/Y23S	455	126.4	145	49.8

Table 3.4. The median fluorescence intensity values (MFI) and percentage values of tetherin-GFP$^+$ cells for analyzing the downregulation of cell surface tetherin expressions by the SRV-3 envelope alanine scanning mutants

Tetherin Antagonist	Human tetherin		Rhesus tetherin	
	MFI	% change in MFI	MFI	% change in MFI
Vector	360	100.0	291	100.0
Vpu	268	74.4	394	135.4
Nef	318	88.3	192	66.0
WT SRV-3 Env	543	150.8	196	67.4
A_3A_4	435	120.8	182	62.5
A_{23}-A_{26}	268	74.4	180	61.9
A_{27}-A_{30}	373	103.6	180	61.9
A_{35}-A_{39}	406	112.8	170	58.4

3.9 SRV-3 envelope trafficking mutants affect viral release in the presence of tetherin

Since the SRV-3 Env trafficking mutants and the alanine scanning mutants did not have any impact on the downregulation of cell surface rhesus tetherin levels, I next wanted to ask whether these mutants affect the ability of the SRV-3 envelope to rescue SIVmac239Δ*nef*Δ*env* virion release from rhesus tetherin. I performed the single-cycle VLP release assay by cotransfecting cells stably expressing human and rhesus tetherin orthologues, with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing either the wild-type SRV-3 envelope or the SRV-3 envelope cytoplasmic tail trafficking mutants (the SRV-3 Env CT- L3S or Y23S or Y35S or L3S/Y23S) or the SRV-3 envelope alanine scanning mutants (A_3A_4 or A_{23}-A_{26} or A_{35}-A_{39} or A_{17}-A_{22} or A_{27}-A_{30}). Stable cells expressing an empty vector were transfected with SIVmac239Δ*nef*Δ*env* proviral plasmid and all SRV-3 Env CT mutants to ensure that these mutants did not cause a general enhancement of viral release in the absence of tetherin. Forty-eight hours post-transfection, transfected cells were harvested and lysed and cultured supernatants were subjected to ultracentrifugation. Expression of the SIVmac239 Gag polyprotein and capsid protein in the cell lysates and the presence of viral capsid protein in the pelleted supernatant was visualized by western blots (Figure 3.9).

Similar to the wild-type SRV-3 envelope, the SRV-3 envelope trafficking mutants and the alanine scanning mutants were unable to rescue virions from human tetherin (Figure 3.9). This finding is consistent with the data from the downregulation assay in which we observed that the mutants also did not affect the cell surface human tetherin levels. In contrast, the envelope trafficking mutants failed to completely rescue virions

from rhesus tetherin and this was very much evident from the band intensity values (Figure 3.9). Compared to the wild-type SRV-3 envelope, mutations of the SRV-3 Env CT dileucine motif (LM to A_3A_4) and the tyrosine motif at position 35 ($Y_{35}LTLT_{39}$ to A_{35}-A_{39}) drastically reduced virion release in the presence of rhesus tetherin. However, the other alanine scanning mutants were able to rescue virions from rhesus tetherin but at levels lower than the amount of virions released in the absence of any tetherin (Figure 3.9). It is possible that although these mutants are able to sequester tetherin in the TGN but they fail to get translocated to the plasma membrane due to misfolding. The other possible explanation is that the SRV-3 envelope mutants might fail to get incorporated into the virions which is affecting the ability to rescue virions from rhesus tetherin. Overall, the data from this experiment suggested that although the SRV-3 envelope cytoplasmic tail mutants did not affect the ability of SRV-3 Env to downmodulate rhesus tetherin from the cell surface but they did affect the ability of the viral envelope to rescue virions from rhesus tetherin.

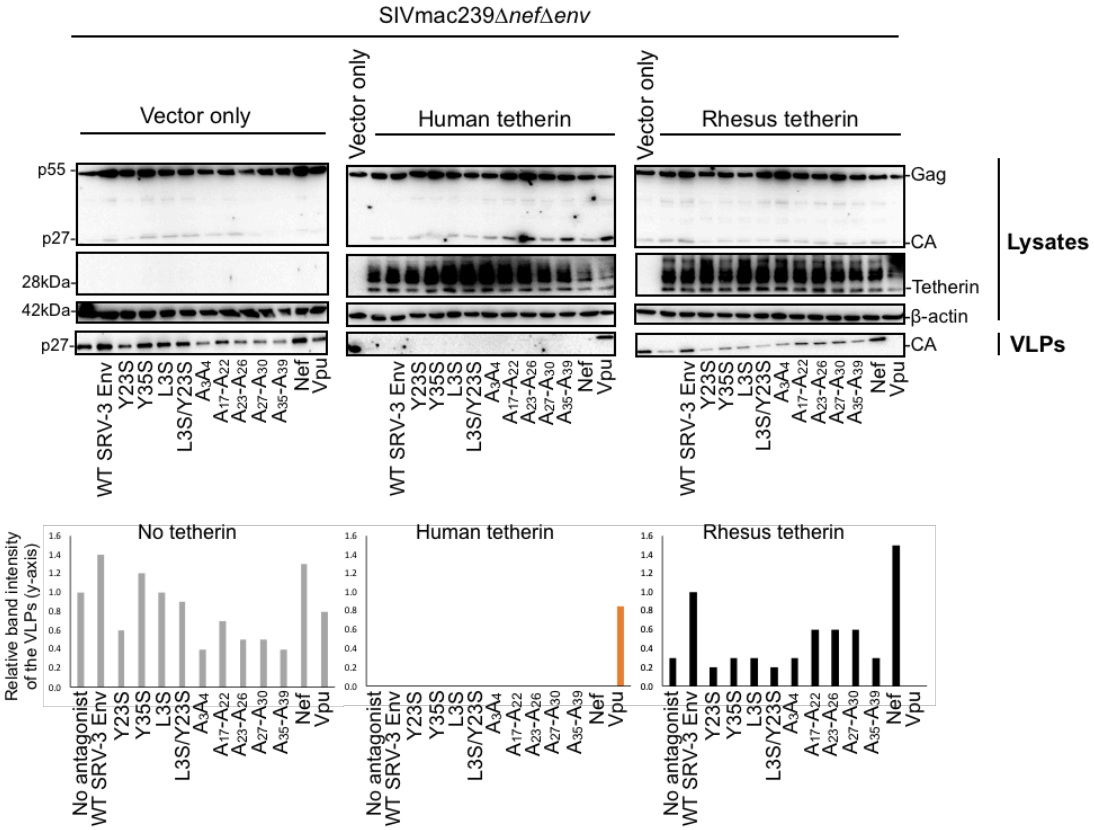


Figure 3.9. SRV-3 envelope trafficking mutants affect viral release in the presence of tetherin

Western blot analysis showing the results of cotransfection of cells engineered to stably express rhesus or human tetherin orthologues, with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing either wild-type SRV-3 *env* or SRV-3 *env* cytoplasmic tail mutants. Stable cells expressing an empty vector, were cotransfected with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing either wild-type SRV-3 *env* or SRV-3 *env* cytoplasmic tail mutants. The SIV Gag and capsid protein expressions were probed with anti-SIVmac p27 monoclonal antibody and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta-actin was used as a loading control for the cell lysates.

3.10 Envelope glycoproteins of retroviruses in the RDR interference supergroup are tetherin antagonists

Simian retroviruses (SRVs) belong to the RD114/D-type (RDR) interference supergroup, that consists of multiple pathogenic simple retroviruses from different genera infecting diverse hosts like carnivores, OWMs, new world monkeys (NWMs) and even birds.[12] Apart from simple exogenous retroviruses, this group also includes a number of endogenous retroviruses. Although these viruses are unrelated across their *gag*, *pro* and *pol* genes, they all share a homologous *env* gene due to recombination. As a result of which, all these viruses use the same receptor (ASCT2) and have significant degree of sequence identity in their SU and TM subunits.[12] I, therefore, wanted to investigate the extent of distribution of tetherin-antagonism among the retroviruses of the RDR interference supergroup. I screened a few representative RDR envelope proteins of SMRV, BaEV, the feline RD114 and REV for their ability to rescue SIVmac239Δ*env*Δ*nef* virions from a panel of tetherin homologs from baboon, squirrel monkey, cat and dog. 293T-based cell lines were engineered to express the tetherin homologs from baboon, squirrel monkey, cat and dog. These stable cells were cotransfected with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing either SIVmac Nef, or HIV-1 Vpu, or SRV-3 envelope, or envelope glycoproteins of SMRV, BaEV, RD114 and REV. As a control, cells stably expressing an empty vector were also transfected with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing either SIVmac Nef or HIV-1 Vpu or SRV-3 envelope or envelope glycoproteins of SMRV, BaEV, RD114 and REV to ensure that these expression constructs did not impact viral release in the absence of tetherin. Forty-eight hours post-transfection, transfected cells

were harvested and lysed and cultured supernatants were subjected to ultracentrifugation. Expression of the SIVmac239 Gag polyprotein and capsid protein in the cell lysates and the presence of viral capsid protein in the pelleted supernatant was visualized by western blots (Figure 3.10). The transient expression of the RDR envelope glycoproteins that we have tested were able to promote virion release in the presence of the rhesus macaque, baboon, squirrel monkey, cat and dog tetherin homologs (Figure 3.10A-D), indicating that the anti-tetherin function is not just restricted to the simian retroviruses of Asian macaques, but is actually wide-spread across the simple retroviruses of the RDR interference supergroup. It is noteworthy that SIVmac Nef also promoted virion release in the presence of baboon, squirrel monkey, cat and dog tetherins, thereby showcasing its anti-tetherin function against these tetherin homologs (Figure 3.10A-D).

Figure 3.10. Envelope glycoproteins of retroviruses in the RDR interference supergroup are tetherin antagonists

(A) Western blots showing the results of cotransfection of cell lines stably expressing human or rhesus or squirrel monkey tetherin orthologues, with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing SRV-3 envelope or HIV-1 Vpu or SIVmac Nef or envelope glycoproteins of RD114 or BaEV or SMRV.

(B) Western blots showing the results of cotransfection of 293T-based stable cell lines expressing baboon or feline or canine tetherin orthologues, with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing SRV-3 envelope or HIV-1 Vpu or SIVmac Nef or envelope glycoproteins of RD114 or BaEV or SMRV.

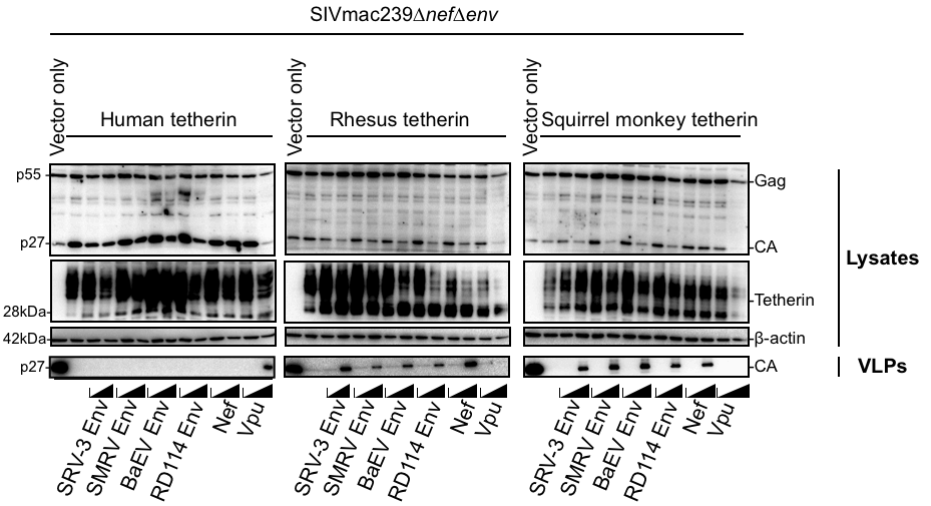
(C) Western blots showing the results of cotransfection of stable cell lines expressing human or rhesus or squirrel monkey tetherin orthologues, with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing SRV-3 envelope or SIVmac Nef or REV envelope.

(D) Western blots showing the results of cotransfection of 293T-based stable cell lines expressing baboon or feline or canine tetherin orthologues, with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing SRV-3 envelope or SIVmac Nef or REV envelope.

(E) Western blots showing the results of cotransfection of an empty vector expressing cell line with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing the SRV-3 envelope or HIV-1 Vpu or SIVmac Nef or envelope glycoproteins of RD114 or BaEV or SMRV or REV.

In figures (A) through (E), the SIV Gag and capsid protein expressions were probed with anti-SIVmac p27 monoclonal antibody and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta-actin was used as a loading control for the lysates.

(A)



(B)

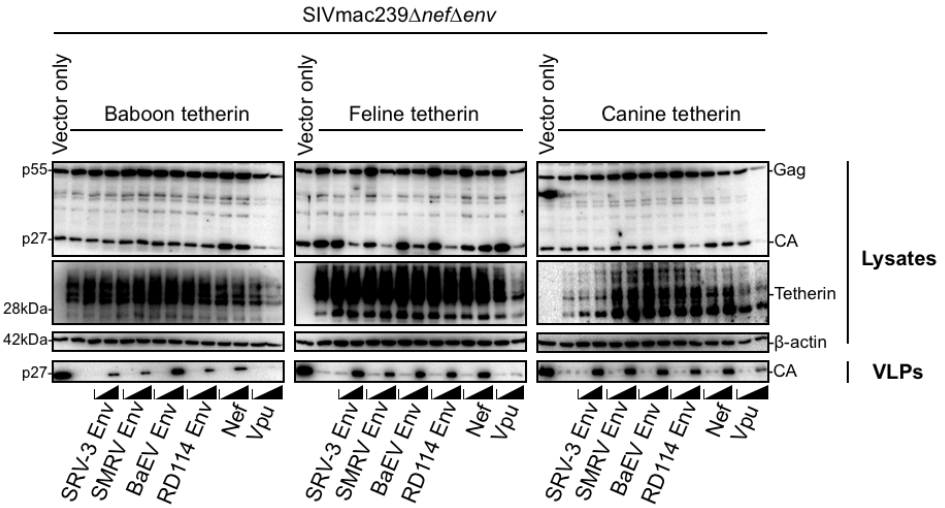
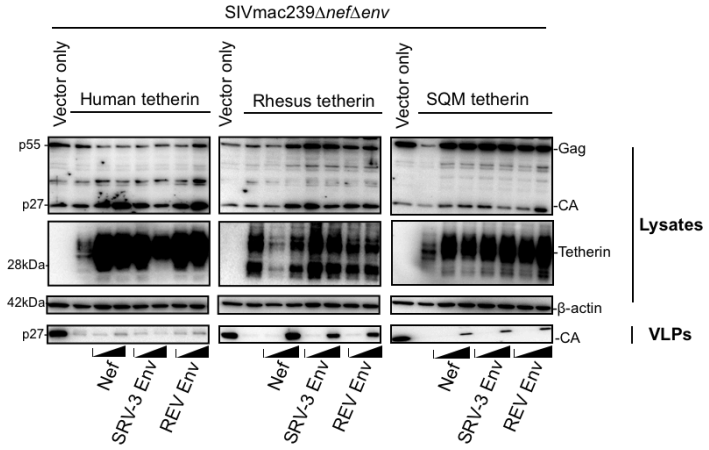
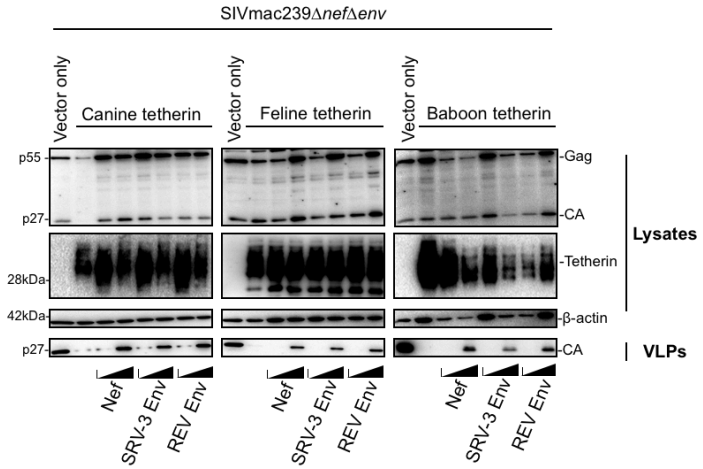


Figure 3.10. Envelope glycoproteins of retroviruses in the RDR interference supergroup are tetherin antagonists. Continued.

(C)



(D)



(E)

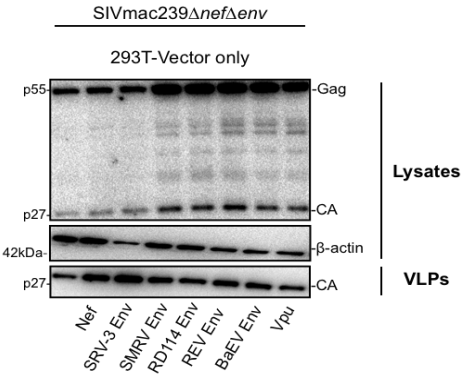


Figure 3.10. Envelope glycoproteins of retroviruses in the RDR interference supergroup are tetherin antagonists. Continued.

3.11 Mutations of aspartate at position 15 in the cytoplasmic tail of rhesus tetherin renders resistance to SRV-3 envelope glycoprotein and Nef

Since this study suggested that both SRV-3 Env and SIVmac Nef are able to counteract tetherin homologs from rhesus macaque, baboon, squirrel monkey, cat and dog, I got interested to examine the determinants in these tetherin homologs that might account for this tetherin-antagonism. Comparative sequence analysis revealed the presence of an aspartate at position 15, that is highly conserved in the cytoplasmic tail of the rhesus macaque, baboon, squirrel monkey, cat and dog tetherin orthologues but absent from human tetherin (highlighted in green in Figure 3.11A). I, therefore generated 293T based stable cell-lines expressing rhesus tetherin point mutants, in which the aspartate at position 15 was either deleted or mutated to an alanine. These stable cell-lines were cotransfected with SIVmac239ΔEnvΔNef proviral plasmid and increasing inputs of plasmids expressing either wild-type SRV-3 Env or SIVmac Nef or HIV-1 Vpu. Consistent with our previous findings, both SIVmac Nef and SRV-3 Env were able to promote virion release and antagonize wild-type rhesus tetherin but not human tetherin. In contrast, Vpu was able to rescue virion release only in the presence of human tetherin and not rhesus tetherin (Figure 3.11B). Mutating the aspartate at position 15 to an alanine or deleting it, rendered rhesus tetherin resistant to both SIVmac Nef and SRV-3 envelope (Figure 3.11B). The transient expression of SIVmac Nef and SRV-3 envelope were unable to rescue virions in the presence of the rhesus tetherin mutants in which the aspartate at position 15 is either deleted or substituted with an alanine. In fact, the amount of the virions rescued by SRV-3 envelope in the presence of the rhesus tetherin mutants were similar to that of the virus restriction observed when SIVmac239Δ*nef*Δ*env* proviral

plasmid was transfected in rhesus and human tetherin expressing cells in the absence of any tetherin antagonist (Figure 3.11B). Therefore, this data indicates that the aspartate at position 15 in the cytoplasmic tail of rhesus tetherin is critical for the tetherin-antagonism of SRV-3 envelope and SIVmac Nef.

3.12 Tetherin-antagonism is a conserved feature of most gammaretroviral envelopes

The RDR envelope resembles a typical gammaretroviral envelope, that is characterized by (i) intersubunit disulfide linkages between the SU and TM subunits and (ii) the presence of a canonical immunosuppressive domain (ISD).[12] Since this study expanded the anti-tetherin function to most RDR envelopes, I got interested to ask whether tetherin-antagonism was a conserved feature across all gammaretroviral envelope. In order to answer this question, I used the envelope glycoproteins of: (a) the most commonly studied exogenous gammaretrovirus, the ecotropic Murine Leukemia virus (MLV), (b) an ancient non-RDR endogenous retrovirus from baboons belonging to the Fc family called Baboon ERV-Fc (Bab-ERV-Fc) and (c) another endogenous gammaretroviral envelope, the HERV-W Syncytin-1. These envelope glycoproteins were expressed in *trans* in the single-cycle VLP release assay to test their ability to rescue SIVmac239Δ*nef*Δ*nef* virion release in the presence of human and rhesus tetherin. The human and rhesus tetherin expressing stable cell lines were cotransfected with SIVmac239Δ*nef*Δ*env* proviral plasmid and differential inputs of plasmids expressing either SIVmac Nef, or HERV-W Syncytin-1, or wild-type BabERV-Fc Env or wild-type MLV Env. SIVmac Nef was used as a control against rhesus tetherin in this experiment.

Forty-eight hours post-transfection, the transfected cells were harvested and the viral protein expression in both the cell lysates and pellets were analyzed by immunoblotting. None of the viral envelope glycoproteins were able to promote virion release in the presence of human tetherin. The Bab-ERV-Fc envelope was sensitive to both human and rhesus tetherin orthologues, and failed to rescue virions in the presence of either of the tetherin orthologues (Figure 3.12). In contrast, the transient expression of ecotropic MLV envelope and HERV-W Syncytin-1 were able to rescue SIVmac239Δ*nef*Δ*env* virions in the presence of rhesus tetherin (Figure 3.12). These finding signify that tetherin-antagonism may be a conserved feature of most gammaretroviral envelopes.

A previous study has shown that the envelope glycoprotein of a *nef*-deleted SIV upon serial passaging acquired anti-tetherin function against rhesus tetherin and that the interaction between the viral Env and rhesus tetherin mapped to the cytoplasmic tails of both tetherin and the antagonist.[159] Since rhesus tetherin was sensitive to the envelope glycoprotein of ecotropic MLV but resistant to the Bab-ERV-Fc envelope, I got curious to investigate whether the cytoplasmic tail of the viral envelope determines sensitivity to rhesus tetherin. A Bab-ERV-Fc envelope chimera was generated in which the native Bab-ERV-Fc cytoplasmic tail was swapped with the ecotropic MLV cytoplasmic tail. This chimeric envelope construct was used in a gain- or loss- of function approach to determine its ability to promote virion release in the presence of tetherin. Similar to the wild-type Bab-ERV-Fc Env, the chimeric Bab-ERV-Fc-MLV CT envelope was unable to rescue viruses from human tetherin (Figure 3.12). However, when the cytoplasmic tail of the Bab-ERV-Fc envelope is substituted with the cytoplasmic tail of the ecotropic MLV envelope, the chimeric envelope gained back its ability to antagonize rhesus tetherin and

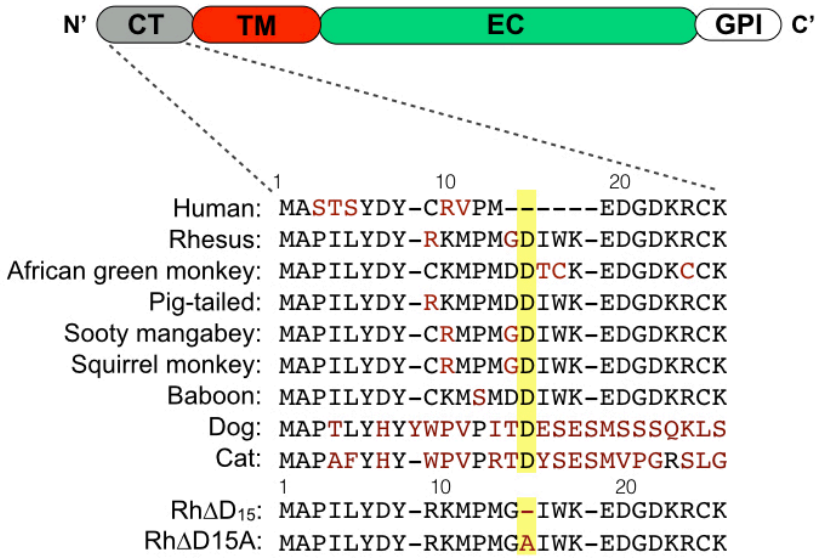
facilitated virion release in the presence of rhesus tetherin (Figure 3.12). The result from this experiment suggest that the cytoplasmic tail of MLV is necessary for evading tetherin-mediated restriction. However, it is still unclear whether any other domains of the MLV envelope glycoprotein might also be critical for tetherin-antagonism.

Figure 3.11: Mutations of aspartate at position 15 in the cytoplasmic tail of rhesus tetherin renders resistance to SRV-3 envelope glycoprotein and Nef

(A) Alignment of tetherin cytoplasmic tail sequences from human, rhesus macaque, pig-tailed macaques, sooty mangabey, squirrel monkey, baboon, cat and dog. The conserved aspartate at position 15 is highlighted in yellow. Dashes indicate deletions or insertions and amino acid differences are highlighted in red. Rhesus tetherin point mutants were created by either deleting the aspartate at position 15 or substituting it with an alanine.

(B) Western Blots showing the results of cotransfection of stable cell lines expressing human or rhesus tetherin orthologues or rhesus tetherin mutants, with SIVmac239Δ*nef*Δ*env* proviral plasmid and wild-type SRV-3 *env* expression construct. The SIV Gag and capsid protein expressions were probed with anti-SIVmac p27 monoclonal antibody and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta-actin was used as a loading control for the cell lysates. When the aspartate at position 15 was either deleted or mutated to an alanine in the rhesus tetherin cytoplasmic tail, SRV-3 Env loses its anti-tetherin activity against rhesus tetherin (highlighted in red boxes).

(A)



(B)

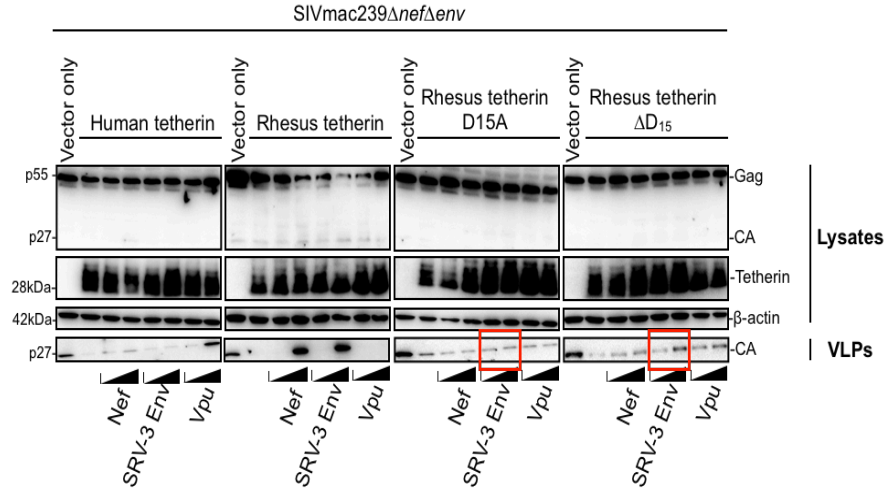


Figure 3.11. Mutations of aspartate at position 15 in the cytoplasmic tail of rhesus tetherin renders resistance to SRV-3 Env and Nef. Continued.

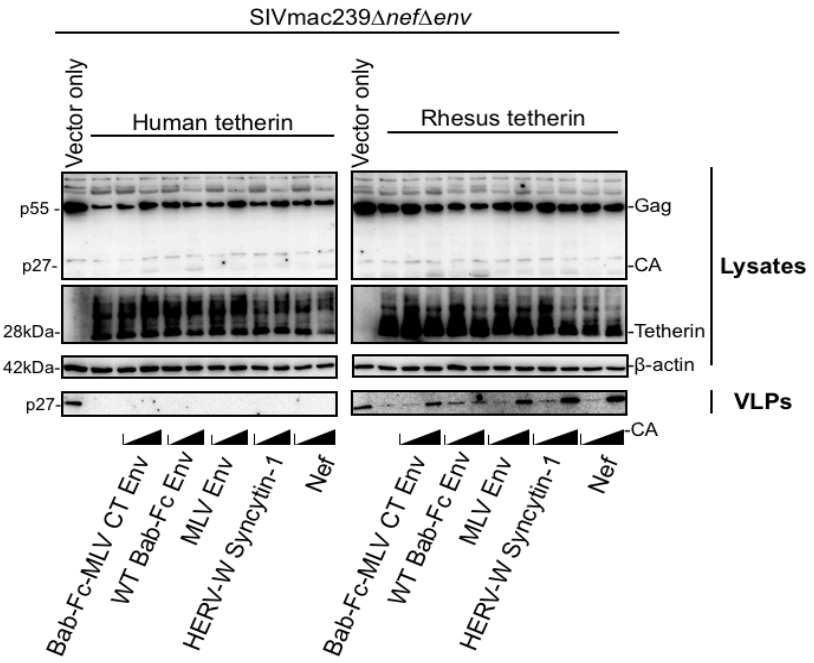


Figure 3.12. Tetherin-antagonism is a conserved feature of most gammaretroviral envelope

Western blots showing the results of cotransfection of human or rhesus tetherin expressing cell lines, with SIVmac239Δ*nef*Δ*env* proviral plasmid and plasmids expressing either HERV-W Syncytin-1 or wild-type BabERV-Fc envelope (Env) or wild-type MLV Env or BabERV-Fc Env/MLV Env chimera. The SIV Gag and capsid protein expressions were probed with anti-SIVmac p27 monoclonal antibody and tetherin expression was probed with rabbit polyclonal anti-BST2 antibody. Beta-actin was used as a loading control for the lysates.

CHAPTER 4: DISCUSSION

During replication in host cells, retroviruses are subjected to restriction by numerous host-encoded factors. These cellular factors commonly known as restriction factors (RFs), inhibit different steps in the virus life cycle to protect the host cell from infection.[36] Consequently, most retroviruses especially the primate lentiviruses have evolved strategies to counteract inhibition by the restriction factors.[43] Retroviruses with complex genomes possess additional accessory genes apart from the four canonical retroviral genes. Many of these accessory genes encode proteins that assist the viruses to evade restriction by the RFs.

Tetherin forms the exit block during retroviral replication, and unlike all other RFs, it restricts the release of a wide range of enveloped viruses in a non-specific manner. Tetherin-evasion mechanisms have only been studied in detail for a few viruses: within the Retroviridae, the focus has been almost exclusively on HIV and SIV, which encode the accessory proteins Vpu and Nef respectively to evade tetherin-mediated restriction.[80,81,112] Thus, there is a wide knowledge gap encompassing numerous simple retroviruses (such as the alpharetroviruses, betaretroviruses and gammaretroviruses) that lack obvious accessory genes and are subjected to restriction by a number of RFs including tetherin during their replication. In this dissertation, I have sought to find an answer to the most obvious and unexplored question: How do simple retroviruses evade restriction by tetherin? Additionally, this study has also helped in delineating one of the several reasons behind the evolutionary success of the gammaretroviral envelope glycoprotein.

I began addressing the question by using SRV-3/MPMV, which is a prototypical type-D betaretrovirus having a very simple genomic organization. This virus was isolated

from the breast carcinoma of a juvenile rhesus macaque and was known to cause severe immunodeficiency in rhesus macaques.[173] Previously, the Bieniasz laboratory reported that SRV-3 was sensitive to restriction by human tetherin.[92] However, given that the natural host of SRV-3 is the rhesus macaque and together with reports that rhesus macaque tetherin is functional against other retroviruses both in vitro and in vivo,[112] I hypothesized that SRV-3 must be resistant to rhesus macaque tetherin. To test this hypothesis, I designed a single-cycle virus release assay to determine the sensitivity of SRV-3 to rhesus macaque tetherin (Figure 3.1A). Our results confirmed that SRV-3 is sensitive to restriction by human tetherin, but also demonstrated that it is resistant to restriction by the tetherin of its natural host, the rhesus macaque (Figure 3.1B). The species-specific differences observed in the pattern of restriction suggested to me that SRV-3 has evolved a mechanism to evade restriction by rhesus macaque tetherin.

Previous studies have shown that some complex retroviruses (e.g., EIAV, HIV-2 and SIV from tantalus monkey) and several unrelated viruses (such as Ebola virus and HSV-1 and -2) use their envelope glycoproteins as tetherin antagonist.[93,101, 158,162,165,166] Since SRV-3 is a simple retrovirus and does not encode any accessory proteins like SIV Nef or HIV-1 Vpu, I hypothesized that the SRV-3 envelope glycoprotein (Env) might have an anti-tetherin function. In the presence of human tetherin, the transient expression of SRV-3 Env failed to rescue the release of the *env*-deleted SRV-3 virions (Figure 3.1C). In contrast, SRV-3 Env expression in *trans* was sufficient to promote the release of the *env*-deleted SRV-3 virions in the presence of rhesus tetherin, suggesting that the SRV-3 Env glycoprotein has anti-tetherin activity (Figure 3.1C).

Unlike the lentiviruses, the assembly of betaretroviruses require the presence of the envelope glycoproteins for efficient viral release.[174] As such, I found it difficult to assess the effects of rhesus tetherin and human tetherin on the release of *env*-deleted SRV-3 virions in the absence of any envelope glycoprotein. Hence, this raises the possibility that the rescue of *env*-deleted SRV-3 virions might be the effect of a general enhancement of virion release by the SRV-3 envelope, and not due to its anti-tetherin function. To address the first possibility, we co-expressed the SRV-3 envelope in *trans* to test if the transient expression of SRV-3 envelope was enhancing the release of the *env*-deleted SRV-3 virions in the absence of any tetherin orthologues. While expression of SRV-3 Env sometimes resulted in a small enhancement of release to controls, this was not sufficient to explain the significant differences in release between cells expressing human tetherin and cells expressing rhesus tetherin. Furthermore, co-expression of VSV-G in *trans* failed to rescue the release of SRV-3Δ*env* virus from either human or rhesus tetherin orthologues (Figure 3.1C), confirming that the efficient release in the presence of tetherin depends on the expression of a functional tetherin antagonist- either Vpu (in the case of human tetherin) or Nef or SRV-3 Env glycoprotein (in the case of rhesus tetherin).

We also observed that the transient expression of SRV-3 envelope rescued a heterologous virus (SIVmac239Δ*nef*Δ*env*) from rhesus tetherin but not from human tetherin (Figure 3.2). These observations strongly suggest that- i) the expression of the SRV-3 envelope alone is sufficient to antagonize rhesus tetherin and does not require the presence of any other SRV-3 proteins and ii) unlike the FIV envelope glycoprotein,

which specifically rescues only FIV particles from tetherin, the anti-tetherin activity of SRV-3 envelope is not specific to SRV-3 virions.[161]

Both HIV-1 Vpu and SIVmac Nef physically interact with their respective host tetherin orthologues.[122,140] Our results from the co-immunoprecipitation assay suggest that the SRV-3 Env physically interacts with rhesus tetherin, and that the interaction involves the $_{14}$GDIWK$_{18}$ residues in the rhesus tetherin cytoplasmic tail. However, it is still unclear whether some or all of these five residues within the $_{14}$GDIWK$_{18}$ motif are critical for mediating the interaction between the SRV-3 envelope and rhesus tetherin.

Interestingly, we found that the SRV-3 envelope glycoprotein exhibits anti-tetherin activity against a diverse panel of tetherin orthologues, including various primate, dog and cat tetherin. While the $_{14}$GDIWK$_{18}$ motif is not absolutely conserved among these tetherin proteins, the aspartate at position 15 is found in all the tetherin orthologues that we tested (except human tetherin). Deleting the aspartate at position 15 or substituting it with an alanine reduced the susceptibility of rhesus tetherin to SRV-3 envelope-mediated tetherin antagonism (Figure 3.11). A similar observation was also reported by Jia et al., regarding SIVmac Nef-mediated tetherin-antagonism.[112] Thus, it may not be the primary sequence but rather a conserved secondary or tertiary structural element encompassing aspartate 15 that confers recognition by the SRV-3 envelope.

Additionally, we observed that the envelope glycoproteins of several additional gammaretroviruses also exhibited patterns of tetherin-antagonism similar to SRV-3 Env (Figures 3.10 and 3.12). All these observations suggest that the gamma-type envelope glycoproteins are the second type of viral protein, after the primate lentiviral Nef proteins, that have converged to specifically target the same element in the cytoplasmic

tail of tetherin for tetherin-antagonism. This convergence further suggests that the region encompassing the $_{14}$GDIWK$_{18}$ motif may have properties especially amenable to protein-protein interactions. Therefore, it would be interesting to find out whether the interactions of tetherin with the gammaretroviral envelope and Nef protein involve similar intermolecular contacts.

The convergence of at least two different viral proteins on the same target also highlights the difficulties of implicating specific viruses as past agents of selection. Previous studies have reported that the loss of the five residues ($_{14}$GDIWK$_{18}$) from the cytoplasmic tail of human tetherin confers resistance against Nef.[89,175] This protective deletion is also present in the archaic humans namely the Neanderthals and Denisova, indicating that the deletion arose nearly 800,000 years ago.[89] Compton et al., have suggested that the variation in this region of tetherin is the result of selective pressure exerted by previous encounters between ancestral humans and lentiviruses that probably encoded Nef-like tetherin antagonists.[175] However, our results suggest that the selection of this protective deletion could also have been driven by an ancient virus with a gamma-type envelope. Indeed, several of the viral Envs used in our study come from viruses that infect primate hosts.

The tetherin antagonists of complex retroviruses such as HIV-1 Vpu and SIVmac Nef downregulate cell surface expression of human and rhesus tetherin respectively.[112] We found that the SRV-3 envelope also downmodulates rhesus tetherin and not human tetherin from the cell surface. However, it is still unclear whether SRV-3 envelope mediates degradation of rhesus tetherin after removing it from the virus budding sites. Thus, further studies are required to define the detailed mechanism of SRV-3 envelope-

mediated tetherin-antagonism.

Previously, a tyrosine-based motifs in the cytoplasmic tail of HIV-2 envelope and the EnvITM of the SIVmacΔ*nefP* were shown to be critical for tetherin-antagonism.[93,159] The cytoplasmic tail of SRV-3 envelope harbors two such tyrosine-based motifs. Of these two motifs, the tyrosine at position 23 is important for envelope trafficking and for the incorporation of the envelope glycoproteins into the virions during viral assembly and budding.[33,176] Additionally, a dileucine-based motif in the SRV-3 envelope cytoplasmic tail has been reported to mediate its anterograde transport.[33] Our data from the site-directed mutagenesis and alanine scanning mutagenesis indicate that the mutations in the SRV-3 envelope trafficking signals did not abrogate its ability to downregulate the cell-surface rhesus tetherin expression (Figure 3.8); however, its ability to rescue virion release in the presence of rhesus tetherin was significantly reduced by the mutations (Figure 3.9). Indeed, reduction in the virion release due to the mutations in the SRV-3 envelope trafficking motifs was observed in the presence as well as in the absence of tetherin (Figure 3.9). These observations corroborate previous findings by Song et al., who showed that the mutations in the tyrosine-based motif at position 23 affected the incorporation of envelope glycoproteins into the SRV-3 virions, and impaired virion assembly and release.[176] Since the mutations in the di-leucine motif of the SRV-3 envelope prevents the anterograde transport of the envelope glycoprotein,[33] it not surprising that these mutations are also leading to a reduction in the virion release irrespective of the presence or absence of tetherin (Figure 3.9). These results also highlight the difficulty in mapping a tetherin-interacting domain in the SRV-3 Env; that is, mapping a binding site by mutagenesis is confounded by the pleiotropic effects of

mutations that may also have on trafficking, assembly and virion release.

Overall, our data suggests that SRV-3 envelope-mediated tetherin antagonism is not dependent on the incorporation of the viral envelope glycoproteins into the virions; instead it indicates the possibility of a direct interaction between the SRV-3 envelope glycoprotein and rhesus tetherin within the secretory pathway preventing tetherin from reaching the cell surface. Hence, we speculate that the SRV-3 envelope might be resulting in the intracellular sequestration of *de novo* synthesized tetherins and preventing their anterograde transport.

Our work reflects that the envelope glycoproteins of gammaretroviruses and the type-D betaretroviruses are potent tetherin antagonists and have evolved to actively evade tetherin-mediated restriction. Several other viral entry proteins have been implicated as tetherin antagonists. For instance, HIV-2 adapted to the deletion in the human tetherin by repurposing its envelope glycoprotein.[93] Similarly, during in vivo passage SIVmacΔ*nefP* also adapted to rhesus tetherin by repurposing its envelope glycoprotein.[159] The entry proteins of Ebola virus (filovirus) and HERV-K (an endogenous retrovirus) have also been described as having anti-tetherin capacity in cell culture.[100,163] To the extent that some or all of these observations reflect bona fide tetherin antagonists, they raise an intriguing question: Do the viral entry glycoproteins have properties that predispose them to evolving anti-tetherin activity?

Similar to several other cell surface and secretory proteins, the viral envelope glycoproteins are synthesized in the RER and traffic through the secretory pathway. Therefore, the viral envelope glycoproteins are in a position to interact with other proteins within the RER and the secretory pathway. Many retroviral envelope

glycoproteins mediate receptor interference by either downmodulating or sequestering their cognate host cell receptors. Therefore, it is easy to imagine that adaptations in a viral entry protein that result in a direct physical interaction between the viral envelope glycoprotein and tetherin within the ER or the secretory pathway could also lead to the intracellular sequestration of newly synthesized tetherins by a mechanism analogous to receptor interference. Since the viral envelope glycoproteins and tetherins are both membrane-associated proteins and localize at the virion budding sites at the plasma membrane, this also suggests the possibility of an interaction between the two proteins at the cell surface resulting in the removal of tetherin from sites of active virion assembly. Hence, considering all these characteristics of the viral envelope glycoproteins, it is not surprising that in the absence of a dedicated tetherin antagonist, the simple retroviruses of the recombinant beta- and gammaretrovirus genera have adapted to evade tetherin-mediated restriction by neo-functionalization of their envelope glycoprotein.

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